THE CARBOHYDRATE CONSTITUENTS OF LUCERNE

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Thesis submitted to the Faculty of Science
of the University of Edinburgh
for the Degree of Doctor of Philosophy

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September, 1960.

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INTRODUCTION

Lucerne (Medicago sativa, L.) otherwise known as alfalfa is a leguminous plant belonging to the same family as beans, peas and clover.

At present no fodder plant is known which can compete with lucerne in nutritive and general importance for feeding to farm livestock. It is one of the most important forage plants in the southern counties of England and areas devoted to its growth have shown considerable increase during recent years.

Lucerne is of particular importance as an independent crop providing a rich source of calcium and protein.

CARBOHYDRATE CONSTITUENTS OF LUCERNE

The carbohydrates which occur in plants can be divided into soluble sugars e.g. mono-, di, and oligosaccharides, reserve polysaccharides and the cell wall polysaccharides.

The monosaccharides glucose and fructose are found in lucerne and of the disaccharides occuring in lucerne, sucrose is the most abundant[1].

It is not unusual to find series of oligosaccharides of increasing chain length in plants. The raffinose family is the most common[2]. The principle members of this series are raffinose, stachyose and verbascose.

$G_{1-2}F$ Sucrose

$Ga_{1-6}G_{1-2}F$ Raffinose

$Ga_{1-6}Ga_{1-6}G_{1-2}F$ Stachyose

$Ga_{1-6}Ga_{1-6}Ga_{1-6}G_{1-2}F$ Verbascose

F = fructofuranose residue G = glucopyranose residue,

Ga = galactopyranose residue.

Raffinose[3] and stachyose[4] have been isolated from lucerne.

The amount of reserve polysaccharide in lucerne is small. In contrast to the grasses[5] it contains no fructosan and only a small amount of water-soluble glucan.

The cell wall polysaccharides of lucerne comprise cellulose, hemicellulose and pectin.

The seeds of lucerne are rich in galactomannan. By extracting lucerne seed with hot potassium hydroxide solution, Hirst and Jones[6] isolated a galactomannan which contained galactose and mannose residues in an unusual ratio of 2:1.

A galactomannan of different composition and structure was isolated by hot water extraction of lucerne seed, by Andrews, Hough and Jones[7]. This polysaccharide contained galactose and mannose residues in the ratio 4:5.

The seasonal variation of carbohydrate constituents of lucerne has been investigated by Hirst, Mackenzie and Wylam[8].

PECTIC SUBSTANCES

Nomenclature and definitions of terms used in pectin chemistry.

Many different terms have appeared in the literature of pectin chemistry. Most have been unnecessary and confusing.

In 1926 a committee of the American Chemical Society attempted to clarify the nomenclature[9]. The nomenclature of pectin chemistry was revised again in 1943[10].

Pectin or Pectic Substance.

The terms applied to the complex colloidal carbohydrates which exist in plants and are composed largely of galacturonic acid units, the carboxyl groups of which may be partially or completely esterified with methanol or forming salts.

Pectic Acid.

A term applied to those pectic substances which are essentially free of methyl ester groups.

OCCURRENCE AND EXTRACTION OF PECTIN

Pectic substances occur widely in the plant kingdom. They are most abundant in soft plant tissues composed mainly of primary cells in stages of rapid growth. In such tissues pectin is found in the primary cell walls and intercellular spaces and some soluble pectin is found in the cell vaccuole.

As plant tissues mature and become liquified the concentration of pectin decreases. The pectin content in woody tissues is low because the volume of pectin containing layers is small. However pectin does

occur in relatively large amounts, (10%) in the inner bark of black spruce[11].

There is doubt about the physiological significance of pectic substances in plants - they are highly hydrophilic. This characteristic suggests that pectin could act as an agent in water balance of soft tissues. It has been suggested that pectin acts as a cement which binds primary cells together. Although a pectin gel has little rigidity, its affinity for water could help to maintain the turgidity of cells which gives soft tissues their necessary rigidity.

Before pectic substances are extracted from plant tissues it is a general practice to denature enzymes by treating the freshly cut plant material with boiling 90% ethanol. The ethanol also dissolves soluble sugars, pigments and waxes.

Subsequent extraction procedures are made more efficient if the plant materials are finely ground.

Extraction with cold water is one of the simplest methods for removing water-soluble pectin. When plant tissues contain a high proportion of water (fruit) the water-soluble pectin can be extracted by grinding the tissues and collecting the juice which will be rich in pectin.

Hot water can be employed to extract pectin but this method can be used with safety only when the heating period is short. Long periods of treatment with hot water tend to cause degradation.

Many agents have been suggested for the extraction of pectic substances but in the light of present knowledge it is apparent that most of these agents can cause substantial degradation.

The use of dilute acid will aid the removal of pectin but hydrolysis of araban associated with pectin is readily achieved under these conditions. If the role of arabinose units in the structure of pectin has to be determined this procedure must be avoided.

Alkaline reagents such as sodium hydroxide, sodium carbonate and ammonium hydroxide have been proposed, but are undesirable for extracting pectin because esterified polygalacturonic acid is susceptible to alkaline degradation[12].

To remove water-insoluble forms of pectin from plant tissues it is necessary to eliminate the calcium and magnesium ions which render pectin insoluble.

An efficient method for doing this is to treat the plant material with hot ammonium oxalate solution. In this way ammonium pectate is formed which is readily soluble in water.

PURIFICATION OF PECTIN

The non-carbohydrate contaminants associated with pectin are generally protein and inorganic substances.

The removal of protein from solutions of pectin has been achieved by using proteolytic enzymes. Another effective method is to render the protein insoluble. Chloroform[13] used in the presence of amyl alcohol will readily precipitate protein.

Similarly trichloroacetic acid[14] will precipitate protein from aqueous solution. Dialysis[15] and electrodialysis[16] have been

used to remove inorganic contaminants from pectin solutions.

By treating a solution of pectin with cation and anion exchange resins in their hydrogen and hydroxyl forms respectively it is possible to reduce the ash content of pectin to negligible proportions[17] (0.1%). The isolation of purified pectin has been achieved by electrodeposition[17]. A carbohydrate contaminant commonly associated with pectin is starch which is readily removed by treatment with amylolytic enzymes.

THE ROLE OF GALACTOSE, ARABINOSE, AND GALACTURONIC ACID IN PECTINS.

It has been known for a long time that galactose, arabinose and galacturonic acid are present in pectic substances and that the relationship between them is very close.

Fractionation of a limited number of pectins has been achieved to a certain extent and as a result it has been widely accepted that pectins are mixtures[18] of three polysaccharides namely galactan, araban, and polygalacturonic acid, though the possibility that complex polysaccharide material exists in pectin has not been overlooked.[19, 20, 39.]

Araban associated with pectin is soluble in 70% ethanol, consequently repeated precipitation of pectin with ethanol tends to remove araban-rich polysaccharide. However co-precipitation is liable to occur. A more effective method for removal of free araban is to reflux the pectin with 70% ethanol.

Hirst and Jones extracted peanut pectin with 70% ethanol for two weeks[21] and isolated araban contaminated with only small proportions of other substances.

Araban acetate prepared from this crude araban was free from galactan and polygalacturonic acid and gave free araban when it was deacetylated.

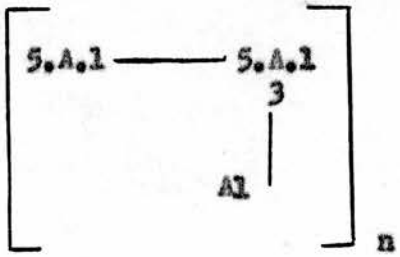
In the same way araban has been isolated from citrus pectin[22].

The araban was found to have a high negative rotation and it was readily hydrolysed under mild conditions to L-arabinose. Consequently it was suggested that the polysaccharide is composed of L-arabofuranose units joined by α linkages.

Study of the methylation products of peanut pectin araban[23] indicated that its components were equimolecular amounts of 2-mono-O-methyl-L-arabinose, 2,3-di-O-methyl-L-arabinose and 2,3,5-tri-O-methyl L-arabinose.

One of the several possible structures of the araban based on methylation study was proposed as a chain of 1-5'α linked arabofuranose units with single arabofuranose units attached to half of these units by 1-3'α links.

A repeating unit of such a structure is shown below.



A = α linked arabofuranose unit.

Methylated araban was isolated from apple pectin[24] after heating the pectin with methyl sulphate and sodium hydroxide. Under these conditions polygalacturonic acid was destroyed.

This methylated araban was the same type as that derived from

peanut pectin.

The alkali-stable araban-rich polysaccharide of sugar beet pectin has been investigated extensively by a number of workers in this field.

Hough and co-workers isolated the polysaccharide by extracting sugar beet with hot lime-water.

Hydrolysis of the polysaccharide gave L-arabinose, D-galactose, L-rhamnose, and D-galacturonic acid in the approximate molecular proportions 21:3:1:5. Smaller quantities of 2-mono-O-methyl-D-xylose, 2-mono-O-methyl-L- fucose, L-fucose, and an aldobiouronic acid of D-galacturonic acid and 2-mono-O-methyl-D-xylose were produced as hydrolysis products.

Attempts to fractionate the polysaccharide into different components failed.

Fractional precipitation of the acetylated araban gave fractions all of which when deacetylated and hydrolysed gave L-arabinose, D-galactose, L-rhamnose and D-galacturonic acid.

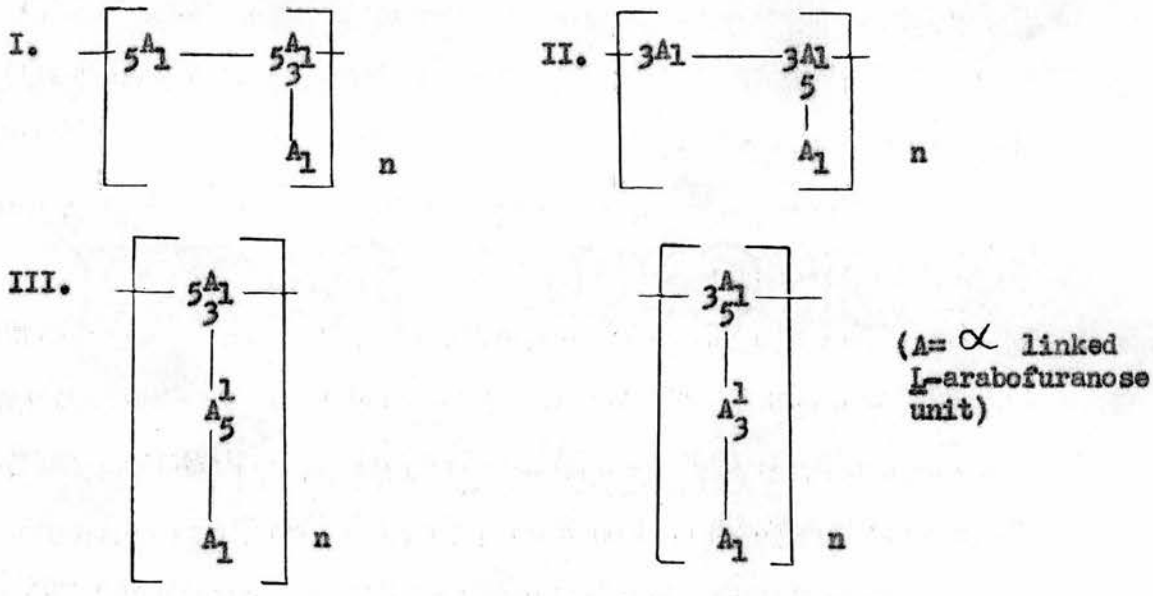
The hydroloysis products of the methylated polysaccharide were investigated[26]. 2-mono-O-methyl-L-arabinose, 2-3-di-O-methyl-L-arabinose and 2,3,5-tri-O-methyl-L-arabinose were identified and smaller amounts of 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose and 2,4-di-O-methyl-D-galactose were isolated and characterised.

The polysaccharide was hydrolysed under very mild conditions (pH 2, at 80° for 6 hrs.). L-Arabinose and only traces of D-galactose were produced. No D-galactosyl-L-arabinose was found.

As a result it appeared that D-galactosyl units are not dispersed on the araban chain but exist as a branched galactan intimately associated with the araban component.

Oligosaccharide fragments have been isolated from the hydrolysis products of sugar-beet araban[27]. Two of these fragments were identified as 5-O-L-arabofuranosyl-L-arabinose and 3-O-L-arabofuranosyl-L-arabinose.

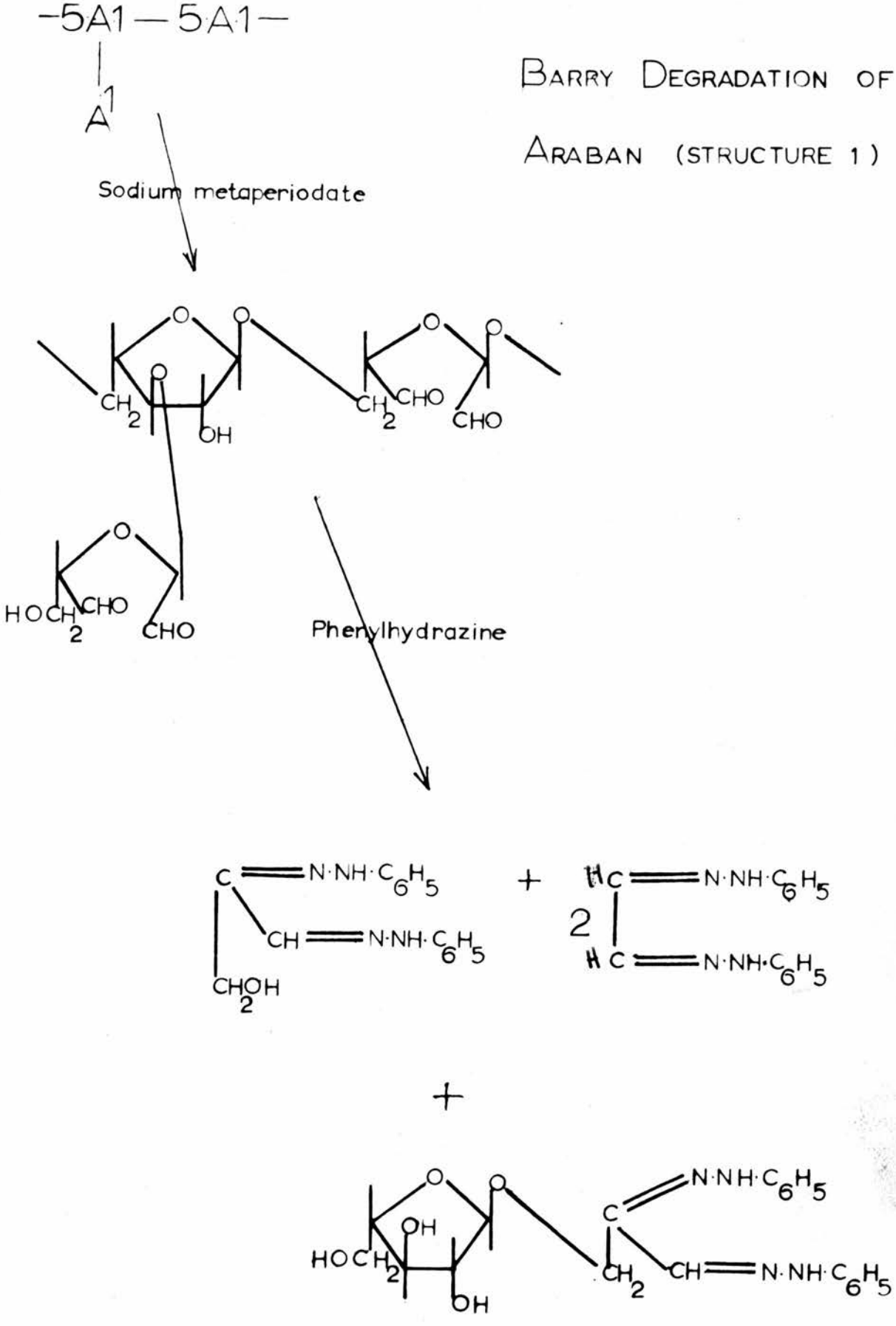
Repeating units of four possible structures based on methylation and hydrolysis studies of the araban are shown below



By applying the Barry degradation[28] to sugar beet araban Finan and O'Colla[29] showed the structure of the polysaccharide is best represented by structures I or II or a ramified modification of these.

The products of the Barry degradation of the araban were glyoxal-bis-phenylhydrazone, glycerosazone and 3-arabofuranosyl-glycerosazone. No arabinosazone or polysaccharide material was detected.

Barry Degradation of Araban (Structure 1)

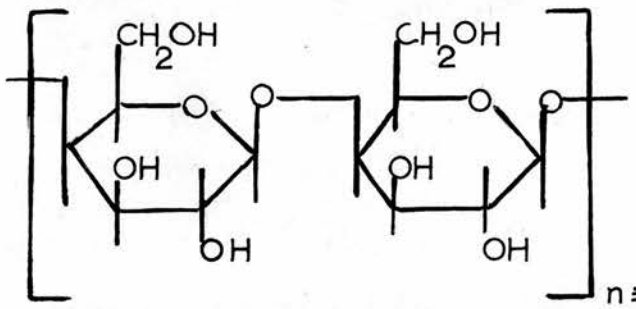


The pectin isolated from white lupin seeds[30] is rich in galactan and it was from this source that Hirst and Jones isolated a pure galactan. This is the only reported instance in which galactan has been isolated from a pectic substance.

The pectin was isolated from the seeds by extraction with hot dilute aqueous sodium hydroxide. Acidic polysaccharide in the pectin was precipitated as the calcium salt and crude galactan remaining in solution was isolated. The crude polysaccharide was methylated and purified by extraction with ether, in which the contaminant, methylated araban, was soluble.

Hydrolysis of the methylated galactan was difficult and required vigorous conditions. The products of hydrolysis were 2,3,6-tri-O-methyl-D-galactose and 2,3,4,6-tetra-O-methyl-D-galactose in the molecular proportion 100:1. The methylated galactan had a low negative optical rotation.

Thus it was suggested that the galactan isolated from lupin seed pectin was a linear chain of β 1-4' linked D-galactose units, as illustrated below.



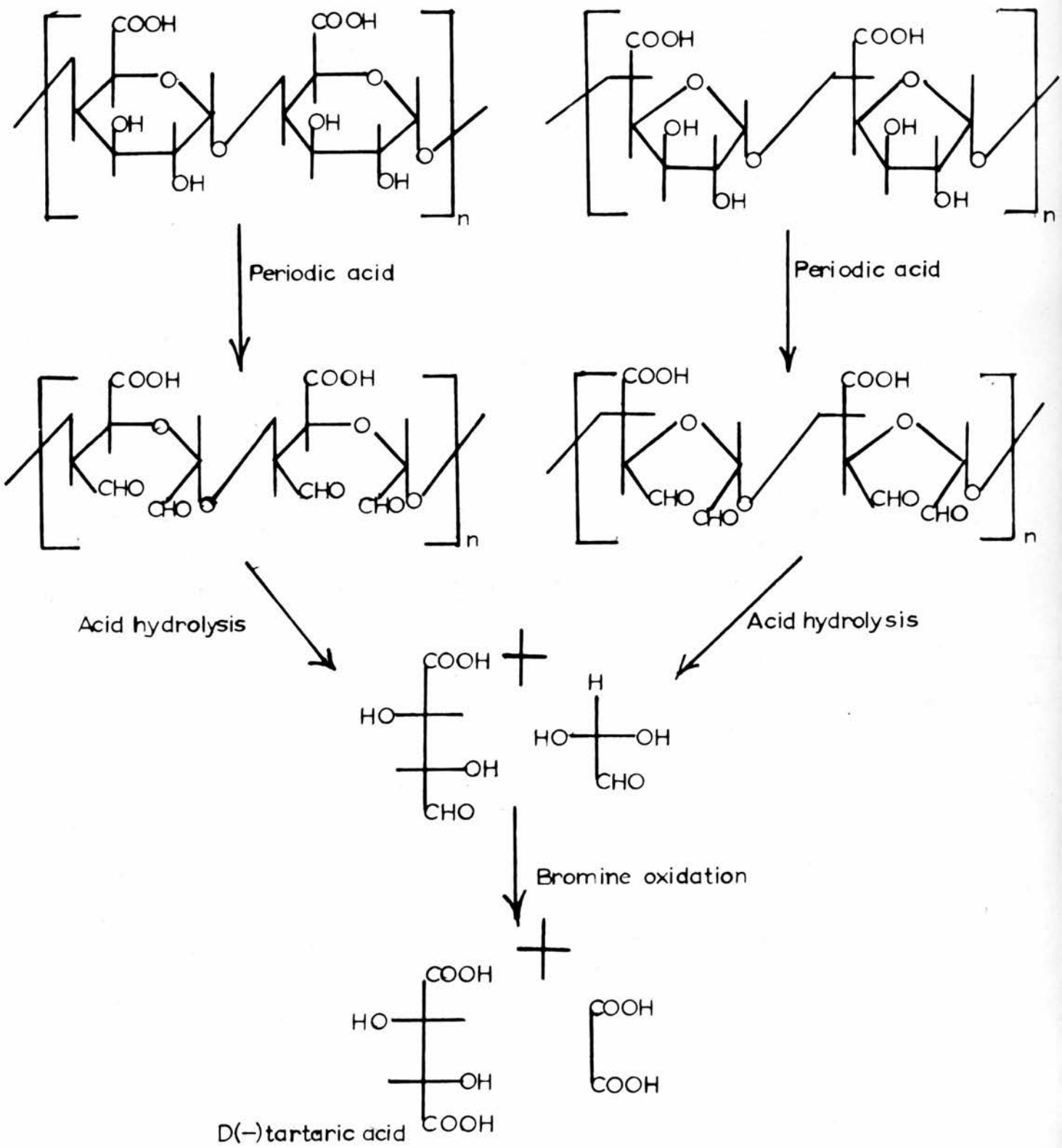
A similar galactan has been isolated from a complex mixture of polysaccharides derived from the seeds of Strychnos nux vomica,[31] and a methylated galactan of this nature has been reported as a minor component in the methylated araban-rich polysaccharide of sugar beet.[26]

Although fractionating of araban and galactan has been achieved from some pectins, it has not been possible to isolate a polygalacturonic acid devoid of neutral sugar residues.

As a result before examining the structure of the polygalacturonic acid chain in pectin, workers in this field have removed associated arabinose and galactose units by acid hydrolysis. The polygalacturonic acid chain is relatively stable to acid hydrolysis. Levene and Kreider[32] were among the first to examine the structure of pectic polygalacturonic acid. Using the technique of periodate oxidation they showed that the galacturonic acid units are linked through carbons C_1 and C_4 and form a long chain. They oxidised polygalacturonic acid with periodic acid and after the product was hydrolysed, aldehydic groups were oxidised with bromine water. A final product was identified as D(-) tartaric acid thus indicating that galacturonic acid units in the pyranose form are linked through carbons C_1 and C_4.

Galacturonic acid units, in the furanose form, linked through carbons C_1 and C_5 would also give D(-)tartaric acid under these conditions.

Levene and Kreider (32) Treatment of Polygalacturonic Acid



Hirst[18], and Jones[33] and Smith[34,35] studied the methylation products of acid-degraded pectins. In all cases methyl 2,3-di-O-methyl-D-galacturonoside methyl ester was obtained as the only product of methanolysis. No methyl 2,3,4-tri-O-methyl-D-galacturonoside methyl ester, which would correspond to non reducing end group, was isolated from the methanolysis product.

The conclusion drawn was that the polygalacturonic acid chain in pectin is composed of 1-4' linked galacturonic acid units and that the proportion of galacturonic acid end group is very small.

A more recent approach to the investigation of the structure of polygalacturonic acid has involved the use of enzymes. Jones and Reid[36, 37, 38] have isolated di- and trigalacturonic acids by enzymic hydrolysis of pectin.

The digalacturonic acid was reduced to a neutral disaccharide which was identified as 4-O-α-D-galactopyranosyl-D-galactose, thus showing that the digalacturonic acid was composed of two D-galactopyranosiduronic acid residues connected by α 1-4' links.

Evidence has been reported which favours the possibility that pectic substances are acidic polysaccharides to which neutral sugars are also glycosidically linked.

It has been shown by methylation study that a pectin isolated from sisal[39] has associated with it no araban of the type found in many other pectins. The arabinose in this pectin occurred entirely as non-reducing end group, and consequently it can be considered that arabinose residues are probably glycosidically linked to the polygalacturonic acid chain.

In this instance two possibilities were suggested (a) that the neutral sugars are constituents of pectin and are linked to the D-galacturonic acid chain or (b) that there is present a mixture of acidic polysaccharides, one composed solely of D-galacturonic acid residues and the other or others containing both neutral and D-galacturonic acid residues.

A chromatographic technique using diethyl amino ethyl cellulose powder in the form of a column has been used to fractionate sugar beet pectin[40]. Neutral polysaccharide composed mainly of arabinose residues was eluted from the column with phosphate buffer (pH 6). Subsequent gradient elution with sodium hydroxide solution (0 - 0.3N) gave acidic polysaccharide fractions. These fractions all contained after hydrolysis, galacturonic acid, arabinose and galactose.

OTHER SUGARS IN PECTIN

In recent years other sugars have been found associated with pectic substances. Their structural significance in unknown.

The separation of minor sugar components from hydrolysates of polysaccharides was difficult before chromatographic techniques were available.

Ehrlich and Haensel[41] claimed that D-fucose is a constituent of pectin isolated from ramie bast. Bauer[42] reported D-xylose as a constitutent of orange pectin and Ehrlich and Schubert[43] reported that D-xylose is a component of flax pectin.

Chromatography has made the isolation of small amounts of sugars associated with pectin possible. Recently L-rhamnose, 2-mono-O-methyl-D-xylose and 2-mono-O-methyl-L-fucose have been reported as constituents

of pectic substances[39,25].

ENZYMIC HYDROLYSIS OF PECTIN

Pectic enzymes[44,45] are divided into two classes, pectinesterases which hydrolyse methyl ester groups, and polygalacturonases which cleave glycosidic links.

The course of action of polygalacturonase on pectic substances has been followed in most cases by estimation of increase in reducing power, by measurement of viscosity changes and by paper chromatography of the products of the enzymic hydrolysis.

The structures of two products of enzymic hydrolysis have been examined by Jones and Reid[36,37]. They used polygalacturonase from Aspergillus foetidus to produce di- and trigalacturonic acids from apple pectin. The digalacturonic acid was shown to be 4-O-α-D-galacturanosyl-D-galacturonic acid. The structure of the trigalacturonic acid was not fully determined but evidence based on methylation study was given which suggested that the structure is one of two types as indicated below.

(a) Gal A 1 - 4 Gal A 1 - 4 Gal A

(b)


(Gal A = α-D-galacturonic acid unit)

Fungal polygalacturonases catalyse the hydrolysis of pectin to galacturonic acid. The rate of hydrolysis is rapid up to 50% hydrolysis and then becomes slower. It is suggested that digalacturonic acid is hydrolysed slowly and that the breakdown of pectin to this product is rapid and accounts for the initial rate of hydrolysis[46]. Di-, tri- and tetra-

galacturonic acids are produced in the initial stages of the hydrolysis.

Evidence has been given which suggests that fungal polygalacturanase is a complex of three or more enzymes[47] and that one enzyme hydrolyses pectin to di- and trigalacturonic acid while another enzyme causes the hydrolysis of di- and trigalacturonic acid to galacturonic acid[48].

It has been reported that yeast polygalacturonase is a single enzyme[49] and that it catalyses the incomplete hydrolysis of pectin to digalacturonic and galacturonic acids, in the ratio of 3:4. Demain and Phaff[50] studied the action of yeast polygalacturonase on di-, tri- and tetragalacturonic acid, and concluded that galacturonic acid end group (it is uncertain which particular end group) strongly inhibits scission of neighbouring glycosidic linkages. Thus digalacturonic acid has little affinity for yeast polygalacturonase.

No reported evidence has been given that acidic oligosaccharides containing neutral sugar residues have been produced by enzymic hydrolysis of pectin. Reid[51] has shown that the majority of fungal polygalacturonase preparations cause the breakdown of pectin to galacturonic acid, arabinose and galactose, and are mixtures of many different polysaccharidases.

D-GALACTURONIC ACID-CONTAINING POLYSACCHARIDES OTHER THAN PECTIN

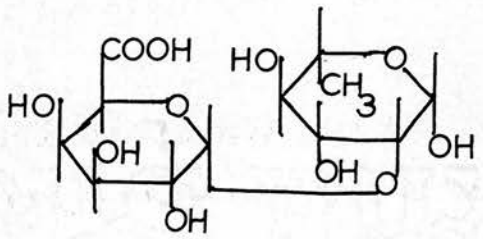
D-galacturonic acid occurs as a component of certain plant gums and mucilages.

Gums[52] of the Khaya, Sterculia and Cochlospermum genera have high D-galacturonic acid content (30-50%). Other sugar residues commonly associated with these gums are D-galactose and L-rhamnose.

Many mucilages[53] contain D-galacturonic acid residues and also

a wide variety of neutral sugar residues, D-xylose, D-galactose, L-rhamnose and L-arabinose occur frequently.

It is interesting that there is a particular structural fragment common to many of the D-galacturonic acid-containing gums and mucilages. This fragment, produced by partial hydrolysis, is 2-O-α-D-galacturonosyl-L-rhamnose



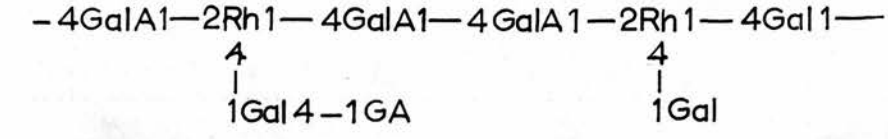
2-O- α -D-galacturonosyl-L-rhamnose

Other residues containing D-galacturonic acid have been isolated from gums and mucilages.

A trisaccharide residue:-

Gal A 1 - 2 Rh 1 - 4 Gal is present in Khaya grandifolia[54] gum.

The main chain of Khaya grandifolia gum contains 1:4 linked D-galacturonic acid residues which are probably of α configuration. Evidence based on methylation and partial hydrolysis studies suggest that some of these units could be adjacent. It is not possible to give a unique formula for the gum but the following structure is not inconsistent with available structural evidence



Two trisaccharide residues, not fully characterised, have been isolated from the partial hydrolysis products of Okra mucilage[55]. Preliminary investigation of these trisaccharides indicated that they are of the form:-

(a) Gal - Gal A - Rh

(b) Gal A - Rh - Gal

KEY

(Gal A = D-galactopyranuronic acid, Gal = D-galactopyranose, Rh = L-rhamnopyranose, GA = 4-O-methyl-D-glucopyranuronic acid).

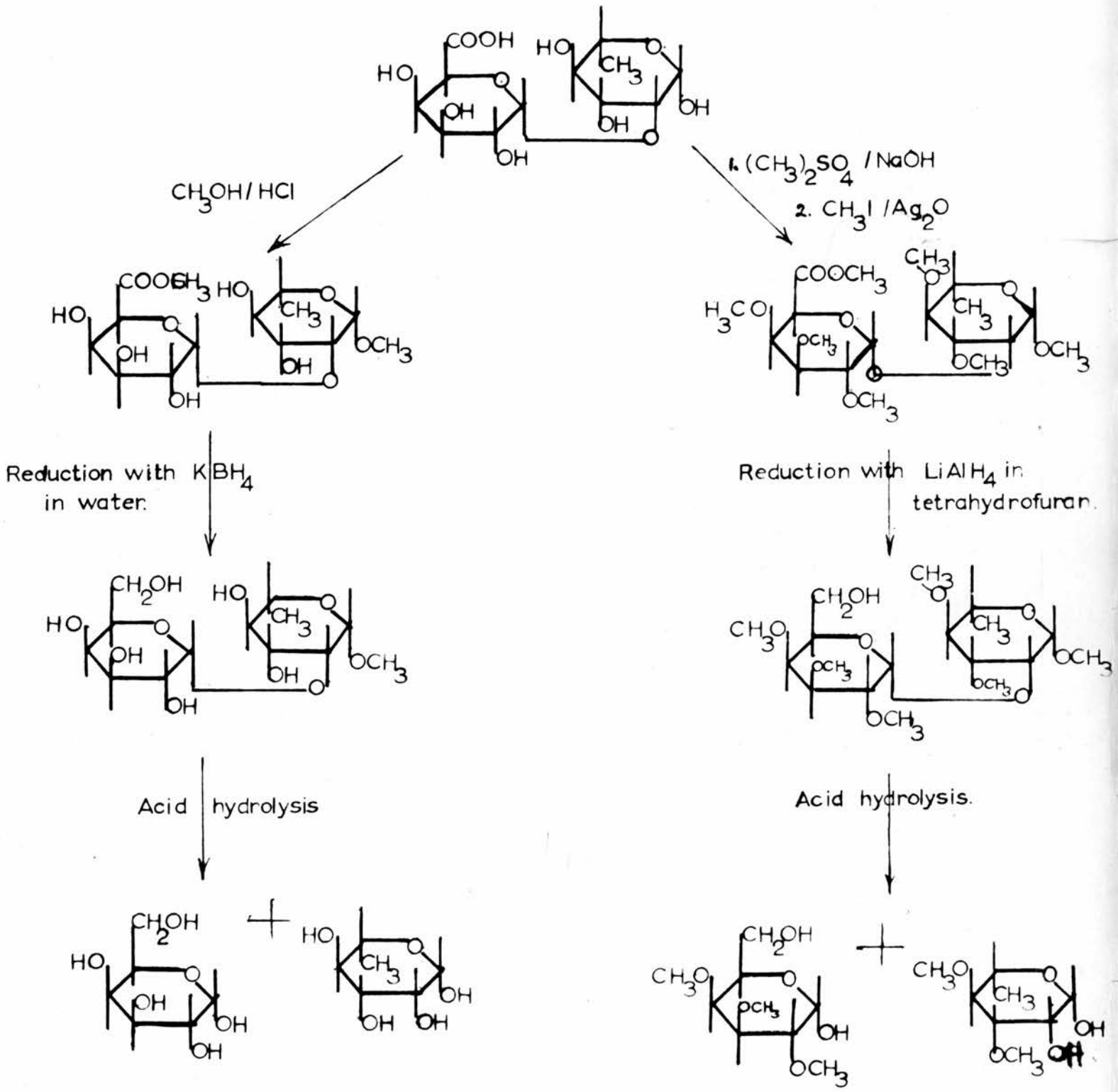
REDUCTION OF ACIDIC CARBOHYDRATE MATERIAL

Reduction with complex metal hydrides has become a valuable technique in carbohydrate chemistry. Many of the problems involved in structural study of carbohydrate materials of high uronic acid content can be overcome by the use of reducing agents.

The carbomethoxy group of uronic esters is reduced rapidly by lithium aluminium hydride[57] and relatively slowly by potassium or sodium borohydride[58]. Lithium aluminium hydride will reduce acid and ester groups but its use is dependent on the substrate being soluble in an ether-type solvent. Reduction in an aqueous solution must be carried out by using sodium or potassium borohydride. Reduction with this reagent is generally incomplete because the alkaline conditions created by these borohydrides cause de-esterification of uronic ester and consequently terminate the reduction. Sodium and potassium borohydrides can be used in buffered solution to avoid this problem[59].

Methyl esters of uronic acids[57], oligosaccharides[60] containing uronic acid and polysaccharides[54] containing uronic acid are reduced by lithium aluminium hydride to the corresponding methylated neutral carbohydrate materials.

Reduction of an Aldobiouronic Acid and its Methylation Product.



Hydrolysis of acidic oligosaccharides and their derivatives requires conditions which involve degradation of uronic acid by decarboxylation. This problem is overcome by reduction of the uronic acid units thus yielding a neutral oligosaccharide which can be hydrolysed relatively easily and is less liable to be degraded under the hydrolysis conditions.

Methylated acidic oligosaccharides and polysaccharides are also resistant to hydrolysis. Reduction not only lowers this resistance to hydrolysis but also by converting methylated hexuronic acid to methylated neutral sugar gives a product which can be identified more readily.

Reduction of high uronic acid-content polysaccharides is difficult. Sisal pectin[39] has been partially reduced by repeated esterification with ethylene oxide and reduction with potassium borohydride. The alkalinity of the solution, by de-esterifying the pectin, is a possible cause of incomplete reduction. There is also a possibility that alkaline degradation of the esterfied pectin could occur under these conditions.

E X P E R I M E N T A L

GENERAL METHODS OF INVESTIGATION

Paper chromatography was carried out on the following Whatman filter papers, unless otherwise stated.

No. 1: A paper with medium flow rate which unless otherwise stated was used for general chromatography of sugars.

No. 3MM: A thick paper with medium flow rate which was used for chromatographic fractionation of sugar mixtures.

No. 31: An acid-washed paper with rapid flow rate which was used for chromatography of acidic oligosaccharides.

No. 31 extra thick: A thick acid-washed paper with very rapid flow rate which was used for chromatographic fractionation of acidic oligosaccharide mixtures.

Chromatography solvent systems (V/V):-

(A) Butan-1-ol: ethanol:water (4:1:5, upper layer).

(B) Ethylacetate:pyridine:water. (10:4:3).

(C) Ethylacetate:acetic acid:formic acid:water (18:3:1:4).

(D) Benzene:ethanol:water. (169:47:5, upper layer).

(E) Ethylacetate:acetic acid:water (3:1:3, upper layer).

(F) Butan-1-ol:acetic acid:water (4:1:5, upper layer).

(G) Ethyl acetate:acetic acid:water (10:5:6).

(H) Butan-2-one:acetic acid:water (9:1:1, saturated with boric acid).

Unless otherwise stated chromatography of methylated sugars was carried out in solvent (A) and R_G values refer to rate of movement relative to 2, 3, 4, 6-tetra-O-methyl-D-glucose in that solvent.

R gal A values refer to the rate of movement of sugars in solvent C relative to D-galacturonic acid.

Chromatography spray reagents

Aniline oxalate (reducing sugars)

Unless otherwise stated chromatograms were sprayed with a saturated aqueous solution of aniline oxalate and developed at 120 - 140° for 2-3 min.

Silver nitrate reagent (non-reducing sugars)

This reagent was used to reveal non-reducing sugars and sugar glycosides. The dried chromatograms were dipped in the silver nitrate reagent (saturated aqueous silver nitrate solution (1 ml.) added to acetone (20 ml.)), dried and then sprayed with ethanolic sodium hydroxide solution (1 pellet of sodium hydroxide dissolved in 0.5 ml. water and diluted to 25 ml. with ethanol.

Brown-black spots appearing rapidly - reducing sugars. Brown-black spots appearing slowly - non-reducing sugars.

To preserve the chromatograms they were washed with 10% sodium thiosulphate solution, washed and dried at 120°.

Urea oxalate (hexuloses).

Fructose and derivatives of fructose were indicated by spraying the chromatograms with saturated aqueous urea oxalate solution and developing at 120 - 140° for 2-3 min. Fructose, free or combined, gave dark blue-grey spots.

Triphenyltetrazolium salt reagent[61] (2-O-substituted reducing sugars)

Chromatograms were sprayed with a 0.5% chloroform solution of

the salt, dried and then sprayed with ethanolic sodium hydroxide solution and heated for a few secs. at 100°. Reducing sugars gave red spots but those in which a substituent was adjacent to the reducing group gave little or no colour. Excess reagent was removed by washing the chromatograms with water.

Ferric chloride/hydroxylamine reagents[62] (Esters and lactones).

The dried chromatograms were sprayed with alkaline hydroxylamine reagent prepared by mixing equal volumes of methanolic N hydroxylamine hydrochloride and methanolic 1.1 N potassium hydroxide and filtered to remove insoluble solids. After 10 min. the chromatograms were sprayed with 1% hydrochloric acid containing 2% ferric chloride. Lactones and esters gave mauve spots.

Periodate/permanganate reagent[63]

Dried chromatograms were sprayed with a mixture of 4 parts 2% sodium periodate with 1 part 1% potassium permanganate in 2% sodium carbonate. On standing at room temperature for about 15 min., sugars (reducing and non-reducing) gave yellow spots on a pink background.

Bromothymol blue indicator (acidic sugars)

Acidic sugars were located by spraying chromatograms with a solution of bromothymol blue (0.1%) in 80% ethanol adjusted to pH 8.

Acidic sugars appeared as yellow spots on a blue-green background.

Chromatograms were allowed to stand in air for at least 24 hr. before spraying.

Removal of acidic sugars from chromatograms was achieved by spraying the chromatograms with the indicator solution, cutting out

areas containing acidic sugars and eluting sugar and indicator from these areas with water. To remove indicator, ethanol (0.2 volume) and charcoal were added to the solutions. The clear solutions were filtered and evaporated to dryness.

Paper ionophoresis. (64,65) was carried out using borate buffer at pH 10. After running for 4-5 hr. at a potential of 500 volts the ionophoretograms were dried and sprayed with aniline oxalate, containing 5% glacial acetic acid.

Cellulose columns were packed dry and washed with water and then solvent. Solvents were purified as follows:-

Light petroleum was shaken with concentrated sulphuric acid washed free of acid and distilled.

Butan-1-ol was refluxed over potassium hydroxide and distilled.

Sugar mixtures other than methylated sugars were freeze-dried onto cellulose powder, packed as a thin layer at the top of the column and then soaked with solvent. The sugars were then eluted with suitable solvent and the eluate was collected on an automatic fraction collector. Every fifth or tenth fraction was examined chromatographically for sugars and fractions containing the same sugars were combined. The resulting solutions were evaporated to dryness and the residues were extracted with ethanol: water and filtered. The filtrates were taken to dryness and weighed.

Charcoal:celite columns were used to separate monosaccharides from oligosaccharides, monosaccharides from methylated sugars and to fractionate methylated sugar mixtures (66,67). Charcoal was washed with water and mixed with an equal weight of celite which had been

previously treated with hot concentrated hydrochloric acid:water (1:1) and washed with water. The mixture was packed as a slurry into columns and washed with water. The sugar solutions were allowed to soak into the charcoal:celite. Monosaccharides were eluted with water. Oligosaccharides and methylated sugars were eluted with a given concentration of either ethanol, or butan-2-one, in water.

Fractionation of methylated sugars on charcoal celite was carried out by gradient elution with water:butan-2-one. Fractions were collected automatically and examined for sugars by chromatography.

Anion-exchange resin columns were used to separate acidic sugars from neutral sugars, and to fractionate acidic sugars.

Amberlite anion exchange resin CG-45 was suspended in water and packed into a column. The column was eluted with 4% sodium hydroxide (6 bed volumes) and then washed with carbon dioxide-free water. To convert the resin to the formate form the column was eluted with 10% formic acid (6 bed volumes) and then washed with carbon dioxide-free water.

The sugar solutions were run onto the column and left to stand overnight. Neutral sugars were eluted with water, and acidic sugars were fractionated by gradient elution with formic acid.

Evaporations were carried out under reduced pressure at 40°.

Small scale hydrolysis was carried out by heating the material (10-20 mg.) with acid (1-2 ml.) at 100°. Using sulphuric acid neutralisation was effected with A.R. barium carbonate. Barium ions were removed with Amberlite resin IR - 120 (H) and the solution was taken

to dryness.

Hydrochloric acid was neutralised with silver carbonate and the silver ions removed with hydrogen sulphide. The solutions were taken to dryness, the residues extracted with acetone:water and the extracts taken to dryness.

The hydrolysates were examined by chromatography.

Methoxyl determinations were carried out by the semi-micro Zeisel method.[68]

Uronic acid determinations were carried out by (a) decarboxylation and titrimetric determination of carbon dioxide released[69] and (b) by the carbazole method.[70]

Optical rotations were observed at 18° ± 2°.

Demethylations[71] were carried out by heating the sugar (5 mg.) with hydriodic acid (1 ml.) in a sealed tube for 5 min. at 100°. The solution was diluted with water and neutralised with silver carbonate. Silver ions were removed with hydrogen sulphide, and the solution was filtered, evaporated to dryness and examined by chromatography.

Nitrogen determinations were carried out by the micro-Kjeldahl method.

Aniline derivatives were prepared by refluxing equimolecular amounts of the sugars with freshly distilled aniline in dry ethanol (5 ml.) for 30 min. with the exclusion of light. The products crystallised on removal of the solvent and were recrystallised from the given solvents.

Aldonolactones were prepared by oxidising the sugar with an excess of bromine water for 24 hr. Bromine was removed by aeration and the solution was neutralised with silver carbonate, treated with hydrogen

sulphide, filtered and evaporated to dryness. The residue was extracted with hot acetone; the extracts evaporated to dryness and the product crystallised from the given solvent.

Preparation of aldonamides. Aldonolactone was treated with dry methanolic ammonia at 0° for 24 hr. The solvent was removed by evaporation and the product recrystallised from the given solvent.

Toluene-p-sulphonyl hydrazones. The sugar (50 mg.) was dissolved in methanol (10 ml.) treated with 7% (W/V) methanolic teluene p-sulphonhydrazine boiling under reflux for 30 min. The reaction mixture was kept at 0° for 20 hr. and the crystalline product isolated by filtration.

2:5-dichlorophenylhydrazones. The sugar was treated with an equal weight of 2:5 dichlorophenylhydrazine in hot methanol containing 0.3% of sodium sulphite, and the whole heated on a water bath until methanol had boiled away. The product was triturated with a little ether, cooled to 0°, filtered and washed with ether. The derivative was recrystallised immediately from a given solvent.

The crude derivatives are light sensitive.

Periodate oxidation of methylated sugars[72]. The methylated sugar (2-3 mg.) was treated with sodium metaperiodate (0.5$\bar{N}$; 0.2 ml.) at 0° for one hr. Ethylene glycol (1 drop) was added, and the solution allowed to come to room temperature. Sufficient sodium hydroxide solution was added to make the solution alkaline to phenolphthalein. The solution was examined chromatographically using solvent A.

The following results were obtained:-

Sugar	Oxidation Products	
	R_F	Colour
2-methyl hexoses 2-methyl pentoses	0.15-0.2	Yellow (methoxymalon-dialdehyde
3-methyl hexoses	ca 0.4-0.5	Pink (2-methyl pentose)
3-O-methyl L-rhamnose	0.67	Brown (bright pink U.V.)
	0.46	Brown (dull yellow U.V.)
	0.2	Yellow (Yellow U.V.)
2,3-di-O-methyl-D-galactose	0.9	Grey
	0.8	Brown
	0.7	Grey

SECTION I.

THE CARBOHYDRATE CONSTITUENTS OF LUCERNE

EXTRACTION OF CARBOHYDRATES FROM LUCERNE

Lucerne which had been oven-dried was extracted with 80% ethanol, cold water, hot water, hot ammonium oxalate solution and hot lime water, in that order.

Alcohol extraction.

The lucerne (2 kg.) was extracted in a Soxhlet apparatus with 80% ethanol. The solution was evaporated to remove ethanol and then water-insoluble material was removed by filtration. The solution was concentrated and clarified by adding equimolecular amounts of cadmium sulphate and barium hydroxide[73] to the hot solution. The insoluble material was filtered off and the filtrate was evaporated to a syrup, which contained mono- and oligosaccharides.

Cold water extraction.

The residue from the alcohol extraction was stirred with cold water for 24 hr. The residue was filtered off and washed with water. The filtrate and washings were concentrated and crude polysaccharide (80 g.) was precipitated by addition of an equal volume of ethanol to the solution. The polysaccharide was dissolved in water, concentrated to remove ethanol and freeze-dried.

Hot water extraction.

The cold water extracted residue was stirred with water at 90° for 3 hr. The extraction was repeated. Polysaccharide (40 g.) was precipitated from the concentrated extract by addition of an equal volume of ethanol. After redispersing the polysaccharide in water, and evaporating the solution to remove ethanol the polysaccharide was freeze dried.

Ammonium oxalate extraction.

The residue from the hot water extraction was extracted twice with 0.5% ammonium oxalate at 80 - 90° for 3 hr. The residue was filtered off and washed. Calcium chloride solution (10%) was added to the extract until precipitation of calcium pectate was complete. The calcium pectate was washed and then suspended in 0.3% ammonium oxalate and heated on a water bath at 90° for ½ hr. Calcium oxalate was removed by centrifuging and ammonium pectate (60 g.) was then precipitated by adding acetone to the solution. The polysaccharide was reprecipitated from 50% acetone and dissolved in water. The solution was concentrated to remove acetone, and freeze dried.

Lime-water extraction.

A portion (1kg.) of the ammonium oxalate extracted lucerne residue was stirred with saturated lime water for 2 hr. at 90°. The solution was filtered, deionised by treatment with Amberlite resins IR - 120 (H) and IR - 4B (OH) and concentrated. Polysaccharide (9 g.) was precipitated from the solution by addition of 8 volumes of ethanol. A solution of the polysaccharide in water was concentrated and freeze-dried.

LUCERNE PECTIC ACID

The freeze-dried ammonium pectate isolated from lucerne by extraction with ammonium oxalate had the following properties.

$[\alpha]_D$ = + 203° (±5°)(C = 0.4 in water)

Ash = 2.8%

Crude Protein = 1%

Uronic acid anhydride = 50% (by decarboxylation)

= 61% (Carbazole method)

A sample of the polysaccharide (20 mg.) was hydrolysed with N-sulphuric acid at 100° for 4 hr. Chromatography, in solvents A and B showed the following neutral sugars:

Sugars	Approximate proportions
Galactose	2
Arabinose	4
Rhamnose	1

also in relatively small amount

Fucose

Xylose

mono-O-methyl pentose (R_G = 0.40)

mono-O-methyl 6-deoxyhexose (R_G = 0.49)

The polysaccharide (20 mg.) was hydrolysed with 2N-sulphuric acid on a boiling water bath for 8 hr. Chromatography of the product showed the sugars indicated above. An increase in the amount of sugar R_G = 0.49 relative to the amount of sugar R_G = 0.40 was apparent

ATTEMPTED FRACTIONATION OF AMMONIUM PECTATE

Precipitation as calcium pectate.

Calcium chloride solution (5%) was added to a solution of ammonium pectate (1%) until precipitation of calcium pectate was complete. The calcium pectate was isolated by centrifugation and washed with water

several times. Ammonium pectate was regenerated by treating the calcium pectate with ammonium oxalate solution (0.3%) for 30 min. at 90°. The solution was centrifuged and ammonium pectate was precipitated by the addition of an equal volume of acetone to the solution. The polysaccharide was freeze-dried.

$[\alpha]_D$ = + 208° ± 5° (C = 1.2 in water)

Uronic acid anhydride = 51% (by decarboxylation)

Chromatography of the hydrolysed polysaccharide showed that the neutral sugars present in the original polysaccharide had been retained.

Precipitation as a complex with cetavlon[74](cetyl trimethyl ammonium bromide).

A solution of ammonium pectate (2%) was added to an equal volume of aqueous cetavlon solution (7%). The precipitated complex was isolated at the centrifuge and washed with water until free of adhering cetavlon. Polysaccharide was recovered by stirring the complex with acetic acid (5N) for 4 hr, and then precipitated by the addition of ethanol. A solution of the polysaccharide was treated with Amberlite resins IR-120(H) and IR-4B (OH) to remove acetate and cetavlon ions. The polysaccharide was freeze-dried.

$[\alpha]_D$ = 204° ± 5° (C = 0.9 in water)

Uronic acid anhydride = 48% by decarboxylation

Chromatography of the hydrolysis product showed galactose, arabinose, rhamnose and other neutral sugars present in the original polysaccharide.

Extraction with 70% ethanol.

Ammonium pectate was dispersed in 70% ethanol and the mixture was refluxed for 4 days. Insoluble material was removed at the centrifuge. The solution was concentrated and then poured into acetone (7 volumes) to

give a precipitate of polysaccharide which represented 0.5% by weight of the original ammonium pectate. Hydrolysis and chromatography showed that it contained galactose, arabinose and rhamnose.

Treatment with saturated lime-water.

Calcium hydroxide was added to a hot solution of ammonium pectate until a little of the alkali remained undissolved. The mixture was stirred at 90° for 1 hr. Insoluble material and solution were separated at the centrifuge.

The solution was deionised with Amberlite resin IR - 120 (H) and concentrated. A small amount of polysaccharide representing less than 1% by weight of the original polysaccharide was precipitated by addition of acetone to the solution.

Chromatography of the hydrolysed product showed that it contained galactose, arabinose and rhamnose.

The water-insoluble material was stirred with 0.5% ammonium oxalate solution for 30 min. at 90°. The mixture was centrifuged, and polysaccharide was precipitated from the solution with acetone.

$[\alpha]_D$ = 206° ± 5° (C = 1.0 in water)

Uronic acid anhydride = 51% (by decarboxylation)

The polysaccharide contained the same neutral sugars as the original ammonium pectate.

Fractionation on a diethylamino ethyl cellulose column[40]

A thin slurry of Whatman diethylaminoethyl-cellulose (30 g.) in 0.5M sodium dihydrogen phosphate buffer (pH 6) was run into a column in small batches, air pressure being applied after each batch was introduced to form a column (30 x 3 cm.) of the cellulose derivative. The column

was eluted with 0.5 M sodium dihydrogen phosphate buffer (pH 6; 1 l.) and then washed with 0.005M sodium dihydrogen phosphate buffer (pH 6; 2l.).

Ammonium pectate (0.6 g.) in water was treated with Amberlite resins IR - 120 (H) and IR-4B (OH) to removed inorganic ions. Then the polysaccharide was freeze-dried, dissolved in 0.005 M sodium dihydrogen phosphate buffer (pH 6; 50 ml.), run on to the column and left to stand overnight.

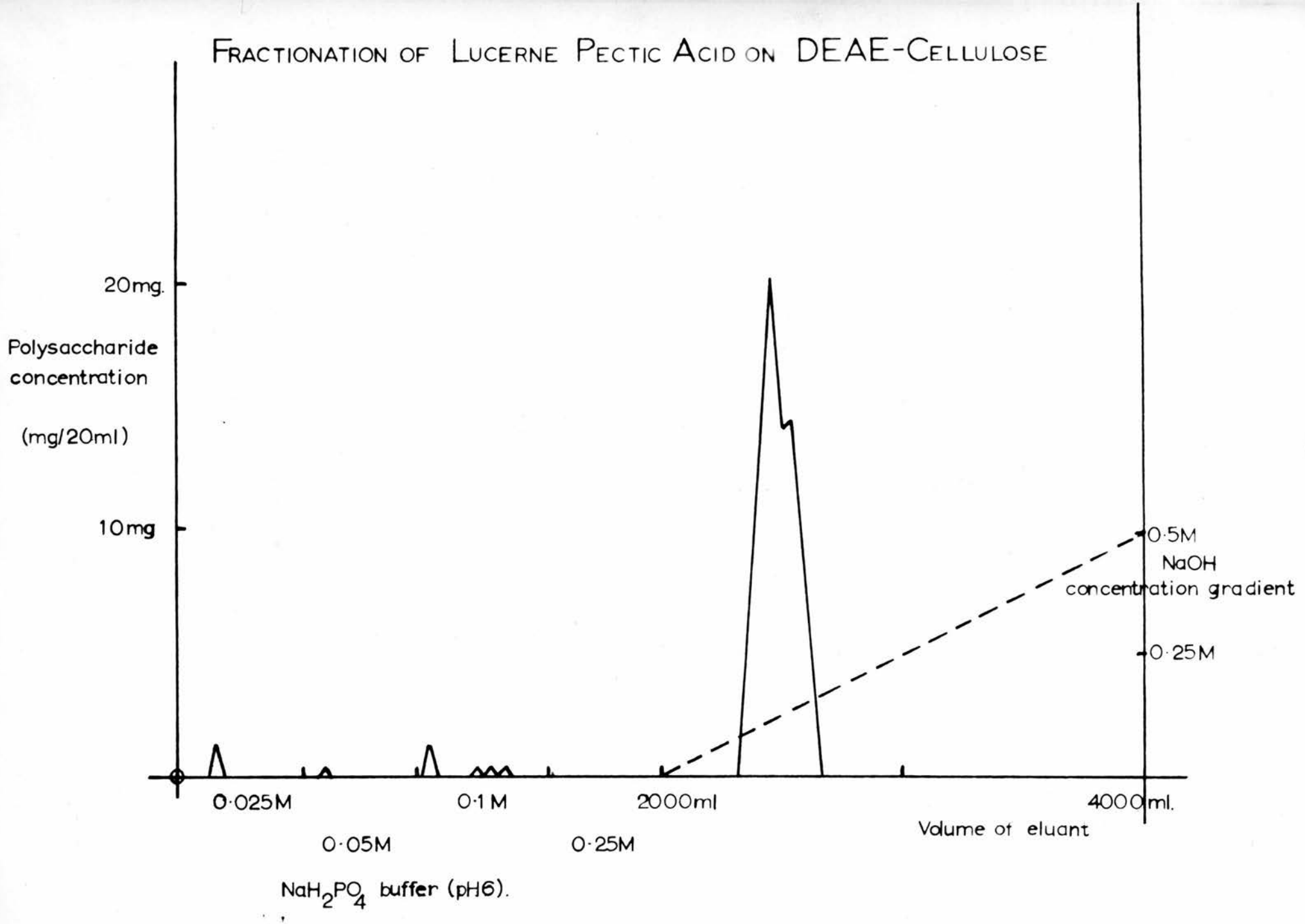
The column was eluted with (a) 0.025 M (500 ml.) (b) 0.05 M (500 ml.) (c) 0.1 M (500 ml.) (d) 0.25 M (500 ml.) sodium dihydrogen phosphate buffer (pH 6).

Fractions (20 ml.) were collected every hour and polysaccharide present in the fractions was determined by the anthrone method using a calibration curve based on L-arabinose as reference sugar[75].

Finally acidic polysaccharide was eluted by gradient elution with sodium hydroxide (0.01 to 0.5 M; 2l.)

The fractions obtained were diluted and acidic polysaccharide was determined by the carbazole method using a calibration curve based on D-galacturonic acid as reference sugar.

A plot of polysaccharide concentration in each fraction against volume of eluant showed that the phosphate buffer eluted a small amount of polysaccharide and that a large amount of acidic polysaccharide was eluted by the sodium hydroxide solution over the concentration range 0.09 - 0.14 M. Fractions containing the acidic polysaccharide were bulked together and stirred into an equal volume of acetone. The precipitated polysaccharide was hydrolysed and chromatography showed that the product contained galactose, arabinose, rhamnose, xylose, fucose, mono-O-methyl pentose and mono-O-methyl 6-deoxyhexose.


FRACTIONATION OF LUCERNE PECTIC ACID ON DEAE-CELLULOSE
20mg.
Polysaccharide concentration
(mg/20ml)
10mg
0·5M
NaOH
concentration gradient
0·25M
0·025M
0·1 M
2000ml
4000ml.
Volume of eluant
0·05M
0·25M
NaH2PO4 buffer (pH6).

HYDROLYSIS OF AMMONIUM PECTATE (I)

Ammonium pectate (6 g.) was dissolved in sulphuric acid (N; 250 ml.) and heated on a boiling water bath for 4 hr. The solution was neutralised with barium carbonate, filtered and concentrated. Barium ions were removed from the solution with Amberlite resin IR-120 (H). The solution was freeze-dried on to cellulose and packed on to a cellulose column (60 x 3 cm.). The column was eluted with butan-1-ol saturated with water. Fractions of eluant containing the same sugars were bulked together and evaporated to dryness.

Fraction 1. (12 mg.)

Chromatography in solvents A and B showed a mixture of three sugars corresponding to rhamnose, mono-O-methyl pentose (R_G = 0.40) and mono-O-methyl 6-deoxyhexose (R_G = 0.49).

Fraction 2. (35 mg.) L-rhamnose

$[\alpha]_D$ = + 8° (C = 1.6 in water)

The sugar was chromatographically pure and identical to rhamnose in solvents A and B.

The syrup crystallised and after recrystallisation from acetone the sugar had m.p. 95 - 98°.

Fraction 3. (56 mg.)

$[\alpha]_D$ = + 82°. (C = 0.6 in water)

Chromatography in solvent A, B and C showed arabinose, xylose and a small amount of fucose.

Fraction 4. (66 mg.) L-arabinose.

$[\alpha]_D$ = + 103° (C = 2.1 in water)

Chromatography in solvents B and C showed arabinose only.

The sugar was characterised as the toluene-p-sulphonylhydrazone derivative m.p. and mixed m.p. 155-6°.

Fraction 5. (62.4 mg.) D-galactose.

$[\alpha]_D$ = + 80° C = 1.8 in water

The sugar was oxidised with concentrated nitric acid on a boiling water bath for 1 hour. On standing overnight the solution gave crystalline mucic acid m.p. = 220°.

HYDROLYSIS OF AMMONIUM PECTATE (II).

Ammonium pectate (20 g.) in sulphuric acid (N; 500 ml.) was heated on a boiling water bath for 3½ hr. Acetone was added to the cooled solution and the precipated acid-degraded pectin was removed by centrifuging, washed with acetone : water (1:1), dissolved in water, concentrated and freeze dried. The solution and washings were evaporated to remove acetone, neutralised with barium carbonate, filtered and concentrated. Barium ions were removed from the solution by treatment with Amberlite resin IR-120 (H). The solution which contained acid and neutral sugars was run on to a column of Amberlite resin CG-45 in the formate form. Neutral sugars were eluted with water; acidic sugars remained on the column.

The neutral sugar solution was concentrated and run on to a charcoal : celite column (30 x 3 cm.) A mixture (A) of neutral sugars was eluted with water and a mixture (B) of two methyl ether derivatives of monosaccharides was obtained by eluting the column with water containing 7% (by volume) butan-2-one.

Monosaccharide mixture (A)

Chromatography showed arabinose, galactose, rhamnose, xylose and fucose.

Monosaccharide mixture (B)

Chromatography of the mixture in solvents A and B showed rhamnose, fucose, mono-O-methyl-pentose and mono-O-methyl-6-deoxyhexose.

The mixture was fractionated on Whatman 3 MM filter paper in solvent A.

Fraction 1. L-fucose

Chromatography in solvents A and B showed fucose only.

The sugar was characterised as the toluene-p-sulphonyl-hydrazone derivative m.p. and mixed m.p. = 169-170°.

$[\alpha]_D$ = -14° ⟶ -8°

Fraction 2.

The sugar was chromatographically identical to rhamnose.

Fraction 3. 2-mono-O-methyl-D-xylose.

R_G = 0.40

$[\alpha]_D$ = +33° (C = 0.60 in water)

Recrystallised from ethanol the sugar had m.p. and mixed m.p. with 2-mono-O-methyl-D-xylose 133-5°.

An X-ray powder photograph of the sugar confirmed its identity.

Fraction 4. 2-mono-O-methyl-L-fucose.

R_G = 0.49

$[\alpha]_D$ = -80° (C = 0.50 in water)

Recrystallised from water the sugar had m.p. 148-151° and m.p. 148-9° when mixed with 2-mono-O-methyl-L-fucose.

An X-ray powder photograph of the sugar confirmed the identity of the sugar.

Acidic sugars.

Acidic sugars were eluted from the Amberlite resin CG-45 column by a gradient of 0 - 2N formic acid. Fractions containing the same sugars were bulked and evaporated to dryness.

Fraction	Wt (g.)	Rgal A.
A	0.65 g.	0.8, 0.47, 0.17, 0.10.
B	0.10	0.85, 0.8, 0.47, 0.17, 0.10.
C	0.32	1.0, 0.10.
D	0.50	1.0
E	0.22	1.0, 0.56.
F	0.50	0.20 (0.5 in solvent G)
G	0.38	0.05 (0.25 in solvent G)

Fractions A and B.

The sugar mixtures were separated on Whatman 31 extra thick filter paper using solvent C for 9 hr. Sugars were located by spraying the chromatograms with bromothymol blue indicator.

Sugar I. (0.412 g.)

Rgal A = 0.80

$[\alpha]_D$ = +84° (C = 4.1 in water)

Hydrolysis of the sugar (5 mg.) with N-sulphuric acid on a boiling water bath for 4 hr. gave galacturonic acid and rhamnose.

The sugar (5 mg.) was treated with 2% methanolic hydrogen chloride in a sealed tube on a boiling water bath for 2 hr. The

product was reduced with potassium borohydride and hydrolysed. Chromatography of the product showed galactose and rhamnose.

The sugar on a chromatogram gave no reaction with triphenyl-tetrazolium salt reagent.

The sugar (0.35 g.) was dissolved in water (20 ml.) and methyl sulphate (30 ml.) and 30% sodium hydroxide (60 ml.) were added dropwise to the solution at 20° over a period of 2 hr. The mixture was stirred for a further 20 hr. The treatment with Haworth's reagents was repeated twice. Finally the solution was heated on a boiling water bath for 20 min., cooled and made slightly acidic with sulphuric acid. The acid solution was extracted at room temperature with chloroform (5 x 100 ml.) and the extracts were dried over anhydrous sodium sulphate and evaporated to a syrup (0.12 g.). The syrup crystallised and was recrystallised from light petroleum (100 - 120°).

m.p. = 67 - 68°

$[\alpha]_D$ = +91° (C = 1.1 in chloroform)

OMe = 40.1 %.

An X-ray powder photograph of the sugar was recorded.

The methylated acid (70 mg.) was dissolved in dry tetra-hydrofuran and an excess of diborane gas (see Section II) was passed into the solution. After 20 hr. excess diborane was destroyed by adding ethanol and water to the solution. Solvent was evaporated and boric acid was removed by repeatedly dissolving the product in methanol and evaporating the solution to dryness. The product was hydrolysed with N-sulphuric acid on a boiling water bath for 4 hr.

Chromatography of the hydrolysate showed a tri-O-methyl-galactose (R_G = 0.68) and a di-O-methyl-rhamnose (R_G = 0.85).

The methylated sugars were separated on Whatman 3 MM filter paper in solvent A.

Fraction (a) 2,3,4-tri-O-methyl-D-galactose (0.031 g.)

R_G = 0.68

$[\alpha]_D$ = +114° (C = 1.0 in water)

The sugar was identified as 2,3,4-tri-O-methyl-D-galactose by conversion to the aniline derivative m.p. and mixed m.p. 163-4°.

Fraction (b) 3,4-di-O-methyl-L-rhamnose (0.029 g.)

R_G = 0.85

$[\alpha]_D$ = +18° (C = 0.30 in water).

Chromatography in solvents A and D showed 3,4-di-O-methyl rhamnose.

The sugar crystallised and was recrystallised from light petroleum (40-60°) - ether m.p. 95-6°.

Sugar 2. (0.040 gm.)

R gal A = 0.47.

$[\alpha]_D$ = -25° (C = 0.43 in water)

Hydrolysis of the sugar (5 mg.) with N-sulphuric acid on a boiling water bath for 4 hr. gave galacturonic acid, fucose and a relatively small amount of other sugars (^{R}galactose = galactose, glucose, arabinose, xylose).

The sugar was treated with 2% methanolic hydrogen chloride in a sealed tube at 100°, reduced with potassium borohydride and hydrolysed. Chromatography of the product showed galactose, fucose, and a relatively small amount of other sugars (^{R}galactose = arabinose,

xylose, glucose).

Chromatography of the sugar in solvent C and spraying the chromatogram with triphenyltetrazolium salt reagent gave an intense red spot at $R_{gal\ A} = 0.47$.

Sugar 3. (0.020 g.)

$R_{galA} = 0.17$

$[\alpha]_D = -8^\circ$ (C = 0.62 in water)

The sugar (5 mg.) was hydrolysed with N-sulphuric acid on a boiling water bath for 4 hr. Chromatography of the product showed galacturonic acid, galactose and a relatively small amount of other sugars ($R_{galactose}$ = arabinose, xylose, glucose).

Methanolysis, reduction with potassium borohydride and hydrolysis gave a product containing galactose and a relatively small amount of other sugars ($R_{galactose}$ = arabinose, xylose, glucose).

Sugar 4. (0.070 g.).

$R_{gal\ A} = 0.10.$

$[\alpha]_D = +82^\circ$ (C = 0.68 in water).

M = 592 (micro-determination by hypoiodite oxidation)[76]

Rhamnose monohydrate under the same conditions gave a value M = 220, corresponding to 91% oxidation of the sugar.

Galacturonic acid anhydride content = 29% (by carbazole method).

Hydrolysis of the sugar (5 mg.) with N-sulphuric acid at 100° for 4 hr. gave galacturonic acid and rhamnose.

Methanolysis, reduction with potassium borohydride and hydrolysis of the sugar gave galactose and rhamnose.

The sugar was treated with bromine water for 48 hr. The product

was hydrolysed and chromatography of the hydrolysate showed rhamnose.

The sugar (10 mg.) was treated with potassium borohydride (10 mg.) in water (1 ml.) for 24 hr. Hydrolysis of the product gave galacturonic acid and rhamnose. Chromatography of the product in solvent H and spraying the chromatogram with the periodate-permanganate spray showed rhamnitol. Chromatography of the product in solvent B and using the silver nitrate reagent spray showed no galactonic acid (as lactone).

The sugar was hydrolysed with N-sulphuric acid at 100° for 1 hr. Chromatography of the hydrolysate in solvent C showed rhamnose, galacturonic acid and a sugar chromatographically identical to 2-O-galacturonosyl-rhamnose.

Fraction C (0.32 g.)

Chromatography in solvent C showed galacturonic acid and a sugar $R_{gal\ A} = 0.10$ isolated from fractions A and B.

Fraction D (0.50 g.)

$R_{galA} = 1.0$

$[\alpha]_D = +58°$ (C = 0.79 in water)

The sugar was characterised as the 2,5-dichlorophenylhydrazone derivative m.p. = 180-2° undepressed on admixture with D-galacturonic acid 2,5-dichlorophenylhydrazone.

Fraction E (0.22 g.)

Chromatography showed galacturonic acid and a relatively small amount of unidentified sugar $R_{gal\ A} = 0.56$.

Fraction F (0.50 g.)

$R_{gal\ A} = 0.2$ (solvent C); 0.50 (Solvent G)

Chromatography of the sugar in solvents C and G showed digalacturonic acid.

The sugar (50 mg.) was neutralised with calcium carbonate and filtered. Ethanol (1.5 volumes) was added to the solution and the precipitated salt was washed with ethanol and ether and dried $[\alpha]_D$ = 114° (C = 1.1 in N-hydrochloric acid).

The sugar (300 mg.) was shaken with benzyl alcohol (30 ml.) saturated with dry hydrogen chloride at room temperature for 24 hr. Ether (35 ml.) was added to the solution. The syrup which precipitated was isolated and dissolved in dry tetrahydrofuran (20 ml.). Lithium aluminium hydride (0.3 g.) was added to the solution and after 2 hr. ethyl acetate and water were added to destory excess hydride. The precipitate (P) was removed by filtration and extracted with hot acetone. The filtrate and extracts were combined and evaporated to a syrup, which was dissolved in ethanol:water (1:4) (20 ml.) containing palladium:charcoal (1:9) (0.1 g.) and shaken in an atmosphere of hydrogen. After 24 hr. the mixture was filtered and the filtrate was evaporated to a syrup which contained galactose and galactobiose.

A further yield of syrup containing galactose and galactobiose was obtained by treating precipitate (P). The precipitate was shaken with Amberlite resin IR-120 (H) in 0.1 N-sulphuric acid. The clear solution was filtered, shaken with Amberlite resin IR-45 (OH) and evaporated to a syrup which was hydrogenated to yield a mixture of galactose and galactobiose.

The total amount of galactose:galactobiose (1:1) mixture was fractionated on Whatman 3 MM filter paper in solvent B.

The isolated galactobiose was chromatographically identical to authentic 4-O- α -D-galactopyranosyl-D-galactose in solvents B and C.

$R_{galactose}$ in solvent B = 0.57.

$R_{galactose}$ in solvent C = 0.29.

The sugar had the same ionophoretic mobility as 4-O-α -D-galactopyranosyl-D-galactose

M_G = 0.38

Fraction G. (0.38 g.)

R_{gal} = 0.25 (solvent G); 0.05 (solvent C)

The sugar (50 mg.) was neutralised with calcium carbonate and filtered. Ethanol (1.5 volumes) was added to the solution and the precipitate was isolated at the centrifuge, reprecipitated twice from 60% ethanol, washed with ethanol and ether, and dried

$[\alpha]_D$ of calcium salt = + 145° (C = 0.36 in N-hydrochloric acid)

ENZYME DEGRADATION OF ACID-DEGRADED PECTIN.

The acetone:water (1:1) insoluble material from the hydrolysis of ammonium pectate with N-sulphuric acid (p. 38) was dissolved in water and adjusted to pH 4 with glacial acetic acid. Light's hemicellulase was added to the solution at 20°. After 8 hr. acetone (1 volume) was added to the solution which was then filtered and evaporated to dryness.

Chromatography of the product in solvents C and G showed galacturonic acid and di- and trigalacturonic acids. No neutral monosaccharides were apparent.

ENZYME DEGRADATION OF PECTIC ACID

Ammonium pectate (15 g.) was dissolved in water (2 l) and passed through columns of IR-120 (H) and IR-45 (OH). Pectinase (2 g.) (Light's) was added to the solution (pH 4) at 20°. After 4 hr. acetone (1 volume) was added to the solution and insoluble material was removed by centrifugation and washed with acetone:water (1:1).

Hydrolysis and chromatography of the hydrolysate in solvents A, B and C showed that this insoluble partially-degraded material contained galactose, arabinose, rhamnose, xylose, fucose, 2-O-methyl-xylose, 2-O-methyl-fucose, 2-O-galacturonosyl-rhamnose and a sugar chromatographically identical to mannose.

The acetone:water solution and washings were evaporated to a syrup (9 g.) which contained monosaccharides, galacturonic acid and di- and trigalacturonic acids.

The syrup (7 g.) was dissolved in water (200 ml.), neutralised with calcium carbonate, filtered and diluted with water. Ethanol (1.5 volumes) was added to the solution. The precipitate was isolated by centrifugation, reprecipitated three times from ethanol:water (1.5:1), dissolved in water and treated with Amberlite IR-120 (H) resin. The solution was evaporated to dryness (Mixture C).

The ethanol:water solution was evaporated to dryness and extracted with methanol under reflux for ½ hr. The methanol solution was evaporated to dryness (Mixture A) and the methanol-insoluble residue was dissolved in water, treated with Amberlite IR-120 (H) resin and the solution was evaporated to dryness (Mixture B).

Mixture A. (1.23 g.)

Chromatography in solvents B and C showed rhamnose, arabinose, galactose and galacturonic acid.

Mixture B. (3.15 g.)

Chromatography in solvents B, C and G showed galacturonic acid and relatively small amounts of arabinose, galactose and digalacturonic acid.

The syrup (3.1 g.) was dissolved in water (100 ml.) and added to an Amberlite CG-45 resin column in the formate form. The column was eluted with water (4 l.) to remove neutral sugars and was then eluted with 0.2 M-formic acid (4 l.). The acid solution was repeatedly concentrated and diluted with water until free of formic acid and was then evaporated to a syrup (2.7 g.).

Chromatography of the syrup in solvents B and C showed galacturonic acid.

The syrup crystallised after being scratched with a drop of glacial acetic acid. The sugar was recrystallised from 95% ethanol.

m.p. = 160-161°

$[\alpha]_D$ = +62° at equilibrium (C = 1.2 in water)

The sugar was converted to the 2,5-dichlorophenylhydrazone derivative which was recrystallised from dioxan; m.p. 180-181° undepressed on admixture with D-galacturonic acid 2,5-dichlorophenylhydrazone.

Mixture C. (2.59 g.)

Chromatography in solvent G showed galacturonic acid and di- and trigalacturonic acids.

The mixture was fractionated chromatographically on Whatman 17

filter paper in solvent G for 8 hr.

Fraction C-1.

Areas of the chromatograms carrying galacturonic acid were eluted with cold water and the eluant was evaporated to dryness (0.84 g.).

Chromatography of the syrup showed galacturonic acid only.

Fraction C-2.

Sugar corresponding to digalacturonic acid was eluted from the chromatograms with hot water in a soxhlet extraction apparatus. The solution was taken to dryness (0.39 g.)

Chromatography of the syrup in solvent G showed digalacturonic acid, galacturonic acid and unknown material $R_{gal\ A}$ = 2.5 in that solvent.

The syrup (50 mg.) was neutralised with calcium carbonate and the calcium salt of digalacturonic acid so formed was precipitated twice from aqueous solution by the addition of ethanol (1.5 volumes). The salt was washed with ethanol and ether, and dried.

$[\alpha]_D$ of calcium salt = + 127° (C = 0.56 in N-hydrochloric acid).

The syrup (0.34 g.) was treated with benzyl alcohol (25 ml), saturated with dry hydrogen chloride, for 24 hr. at room temperature. The solution was shaken with Amberlite resin IR-45 (OH), mixed with a large volume (2 l.) of water and evaporated to dryness.

The product was dissolved in tetra hydrofuran (20 ml.) and lithium aluminium hydride (0.3 g.) was added in small batches to the solution. After 2 hr. ethyl acetate and water were added to destroy excess hydride and the solution was separated from a precipitate at the centrifuge. The solution was treated with Amberlite resins IR-120 (H) and IR-45 (OH) and evaporated to dryness. The product was dissolved in ethanol: water (1:1) containing palladium:charcoal (10%; 0.3 g.) and the mixture was shaken in an atmosphere of hydrogen for 24 hr. The mixture was filtered and evaporated to dryness. Chromatography of the product showed galactose and galactobiose. The galactobiose was isolated by chromatography on Whatman filter paper in solvent B.

The galactobiose was chromatographically and ionophoretically identical to 4-O- α -D-galactopyranosyl-D-galactose

$R_{\text{galactose}}$ in solvent B = 0.57

$R_{\text{galactose}}$ in solvent C = 0.29

M_G = 0.38.

METHYLATION OF AMMONIUM PECTATE

Methyl sulphate (250 ml.) and sodium hydroxide (110 g.) in water (250 ml.) were added dropwise to a solution of ammonium pectate (10 g.) in water (150 ml.) under nitrogen; the temperature was maintained below 30° and the mixture was stirred continuously.

Three further additions of Haworth's reagents were made on three successive days.

The solution was partially neutralised (pH 8) with acetic acid and it was then heated on a boiling water bath for 1 hr. The solution was dialysed against tap water and concentrated to 100 ml. The methylation was repeated.

OMe = 21%

The partially methylated sodium pectate in water (500 ml.) was passed through a column of Amberlite resin IR-120 (H) and the effluent was neutralised with silver oxide and evaporated to dryness.

The dried silver salt (7 g.) was refluxed with methyl iodide (100 ml.) for 2 hr. Silver oxide (8 g.) was added in small batches to the refluxing mixture over a period of 4 hr. The solution was filtered and the solid residue was extracted with hot chloroform (3 x 50 ml.). Evaporation of the solution and extracts gave a crisp solid.

OMe = 34%

Three further treatments with Purdie's reagents were made to obtain methylated methyl pectate (3.6 g.).

OMe = 39%

$[\alpha]_D$ = 133° (C = 0.85 in chloroform)

METHANOLYSIS, REDUCTION AND HYDROLYSIS OF METHYLATED METHYL PECTATE.

Methylated methyl pectate (3.5 g.) was dissolved in 4% methanolic hydrogen chloride (100 ml.) and heated at 100° for 5 hr. in a sealed Carius tube. The solution was neutralised with silver carbonate, filtered and evaporated to a syrup. The syrup was dissolved in dry tetrahydrofuran (100 ml.) and heated under reflux. Lithium aluminium hydride (2 g.) in tetrahydrofuran (50 ml.) was added dropwise to the boiling solution over a period of 1 hr. After 1 hr. the solution was cooled and excess hydride was destroyed by the addition of ethyl acetate. Finally water was added to the solution. The insoluble material was removed by centrifugation and extracted with ethanol and acetone.

The solution combined with the extracts was evaporated to small volumes (150 ml.) treated with Amberlite resins IR-120(H) and IR-4B (OH), and concentrated to a syrup. The syrup was dissolved in N-hydrochloric acid (100 ml.) and heated on a boiling water bath for 5 hr. The solution was neutralised with silver carbonate, filtered, treated with hydrogen sulphide, filtered and concentrated to a syrup (3 g.) which contained methylated sugars.

ISOLATION AND IDENTIFICATION OF METHYLATED SUGARS

The syrup was fractionated by chromatography on a cellulose column (70 x 3.5 cm.) using light petroleum (60-80°) : butan-1-ol (7:3)

saturated with water as eluant.

Fraction 1. R_G = 1.02 (6 mg.)

Chromatography in solvent A indicated 2,3,4-tri-O-methyl rhamnose.

Fraction 2. R_G = 0.98, 0.90 (190 mg.)

Chromatography in solvents A and B showed three components corresponding to 2,3,5-tri-O-methyl arabinose, 2,3,4,6-tetra-O-methyl galactose and 3,4-di-O-methyl rhamnose.

A fourth component was shown by hydrolysis and chromatography to yield 2,3-di-O-methyl galactose.

The syrup was hydrolysed with N-hydrochloric acid at 100° for 4 hr. and fractionated on a cellulose column (50 x 1.6 cm.) using light petroleum (60 - 80°) : butan-1-ol (9:1) saturated with water as eluant.

Subfraction 2a. 2,3,5-tri-O-methyl-L-arabinose (61 mg.)

R_G = 0.96

$[\alpha]_D$ = -40° (C = 1.7 in water)

Chromatography in solvent A gave a grey spot (pink in ultra violet light) corresponding to 2,3,5-tri-O-methyl arabinose.

The sugar was characterised by conversion to the amide of the aldonic acid m.p. = 130 - 132° undepressed on mixing with 2,3,5-tri-O-methyl-L-arabon amide.

Subfraction 2b. 2,3,4,6-tri-O-methyl-D-galactose (42 mg.)

R_G = 0.91

$[\alpha]_D$ = +108° (C = 0.82 in water)

Chromatography in solvent A showed 2,3,4,6-tetra-O-methyl

galactose.

A crystalline aniline derivative was prepared and recrystallised from ethanol m.p. and mixed m.p. with 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, 177-9°.

Subfraction 2c. 3,4-di-O-methyl-L-rhamnose (35 mg.)

R_G = 0.89

$[\alpha]_D$ = +19° (C = 1.56 in water).

Chromatography in solvents A and B showed 3,4-di-O-methyl rhamnose.

Paper ionophoresis showed one component corresponding to 3,4-di-O-methyl-rhamnose.

A lactone was prepared m.p. = 76-77° undepressed on admixture with authentic 3,4-di-O-methyl-L-rhamnonolactone.

Subfraction 2d. (40 mg.)

R_G = 0.51

$[\alpha]_D$ = +79° (C = 1.1 in water)

Chromatography in solvent A showed 2,3-di-O-methyl-galactose.

Fraction 3. (173 mg.)

The syrup (3 mg.) was hydrolysed with N-hydrochloric acid at 100° for 4 hr. Chromatography of the product showed:-

3,4-di-O-methyl-rhamnose (R_G = 0.90)

2,3,4-tri-O-methyl-galactose (R_G = 0.72)

2,3-di-O-methyl-arabinose (R_G = 0.69)

2,3-di-O-methyl-galactose (R_G = 0.50)

2-O-methyl-galactose (R_G = 0.33)

3-O-methyl-galactose (R_G = 0.30)

The syrup (170 mg.) was hydrolysed and the product was fractionated on a cellulose column (50 x 1.6 cm.). The column was eluted with light petroleum (60 - 80°) : butan-1-ol (4:1) saturated with water and finally with water.

Sub-fraction 3a (16 mgm.)

R_G = 0.89

$[\alpha]_D$ = +17° (C = 0.90 in water)

Chromatography in solvents A and D showed one component corresponding to 3,4-di-O-methyl-rhamnose.

Subfraction 3b. (86 mg.)

R_G = 0.71, 0.68

Chromatography in solvents A and B showed 2,3,4-tri-O-methyl galactose and 2,3-di-O-methyl-arabinose in approximately equal amounts.

Subfraction 3c. (45 mg.)

R_G = 0.50, 0.33, 0.30

Chromatography in solvent A showed three components 2,3-di-O-methyl-D-galactose, 2-O-methyl-galactose and 3-O-methyl-galactose in the approximate ratio 3:1:1.

Fraction 4. (264 mg.)

R_G = 0.71, 0.68, 0.63.

Demethylation gave galactose, arabinose and a small amount of rhamnose.

Chromatography in solvents A, B and D showed 2,3,4-tri-O-methyl-galactose, 2,3-di-O-methyl arabinose and a group of components in the region R_G = 0.61 - 0.63.

Partial fractionation of the mixture was achieved on Whatman 3MM filter paper in solvent B.

Sub-fraction 4a (170 mg.)

R_G = 0.71, 0.68.

Demethylation gave galactose and arabinose.

Chromatography in solvent A showed a two component mixture. Isolation of small amounts of two chromatographically pure sugars was achieved on Whatman 3MM filter paper in solvent A.

Sugar (a) 2,3,4-tri-O-methyl-D-galactose

R_G = 0.71

$[\alpha]_D$ = +110° (C = 0.2 in water)

The sugar was converted to the aniline derivative which was recrystallised from acetone; m.p. 162-3° undepressed on admixture with 2,3,4-tri-O-methyl-N-phenyl-D-galactosylamine.

Sugar (b) 2,3-di-O-methyl-L-arabinose.

R_G = 0.68

$[\alpha]_D$ = +95° (C = 0.4 in water)

The sugar was converted to the amide of the aldonic acid and recrystallised from acetone. M.p. 155-6° undepressed on admixture with 2,3-di-O-methyl-L-arabonamide.

Subfraction 4(a) was examined by Dr. C.T. Bishop (77) by vapour phase chromatography.

Chart I shows a vapour phase chromatogram of the methyl glycosides of the components of the fraction. The interpretation of the chart by Dr. C.T. Bishop is given below.

CHART I (Interpretation)

Peak	Inference
1	Possibly methyl-3,5-di-O-methyl-α-L-arabinoside
2	Possibly methyl-3,5-di-O-methyl-β-L-arabinoside
3, 4, 5	All three peaks correspond to 2,3-di-O-methyl-L-arabinose
6, 7, 8	Unknown. Shoulder on the front of peak 8 is methyl-2,4,6-tri-O-methyl-β-D-galactoside
9	Methyl-2,4,6-tri-O-methyl-α-D-galactoside
10	Methyl-2,3,4-tri-O-methyl-β-D-galactoside
11	Methyl-2,3,4-tri-O-methyl-α-D-galactoside

The ratio of methyl-2,3,4-tri-O-methyl-D-galactoside to methyl-2,3,-di-O-methyl-L-arabinoside, determined as the ratio the area of peaks 3, 4 and 5 to the area of peaks 10 and 11, was 1 : 1.13.

Chart II shows a vapour phase chromatogram of the components of sub-fraction 4(a) after complete methylation.

CHART II (Interpretation)

Peak	Inference
1	Methyl-2,3,5-tri-O-methyl-α-L-arabofuranoside
2	Methyl-2,3,5-tri-O-methyl-β-L-arabofuranoside
3	Methyl-2,3,4-tri-O-methyl(α+β)-L-arabopyranoside
4	Unknown
5	Methyl-2,3,4,6-tetra-O-methyl-β-D-galactopyranoside
6	Methyl-2,3,4,6-tetra-O-methyl-α-D-galactopyranoside
7	Identical to peak 8 on Chart I.

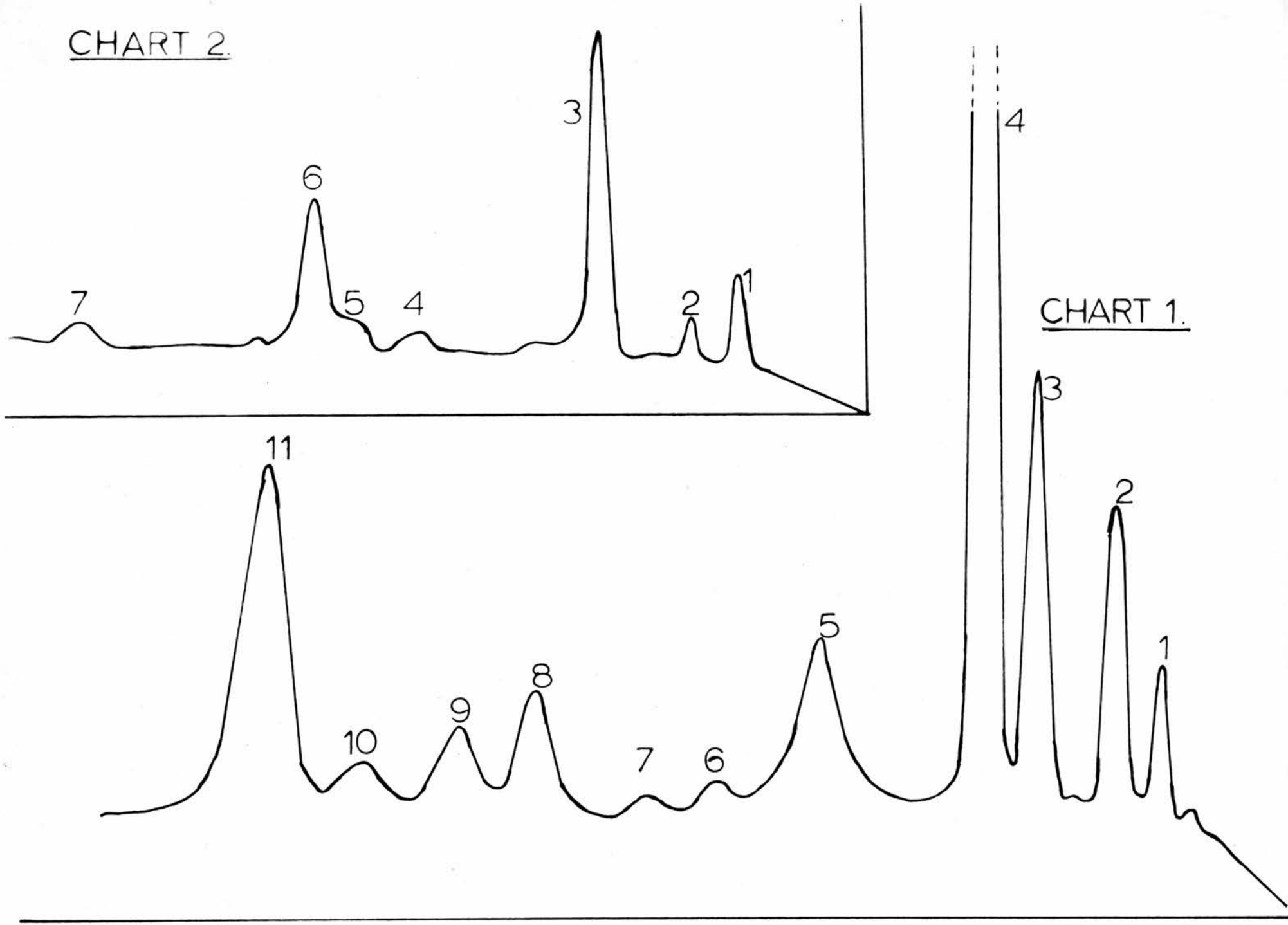


CHART 2.
7
6
5
4
3
2
1
CHART 1.
11
10
9
8
7
6
5
4
3
2
1

Subfraction 4(b)

Chromatography in solvent D showed four components R_F = 0.14, 0.11, 0.09 and 0.05.

The sugar R_F = 0.05 was incompletely separated from the mixture on Whatman 3MM filter paper in solvent D.

R_G = 0.60 $[\alpha]_D$ = +33° (C = 0.27 in water).

Periodate oxidation and chromatography of the oxidation products indicated 3-O-methyl rhamnose.

Fraction 5. (60 mg.)

$[\alpha]_D$ = 62° (C = 1.86 in water)

Chromatography in solvents A and D showed 2,3-di-O-methyl-arabinose, 3-O-methyl rhamnose and 2,3-di-O-methyl-galactose.

Fraction 6. (300 mg.)

R_G = 0.60, 0.52.

Chromatography showed 3-O-methyl rhamnose and 2,3-di-O-methyl galactose in the approximate ratio 1 : 5.

The mixture was fractionated on Whatman 3MM filter paper in solvent A.

Subfraction 6(a)

$[\alpha]_D$ = +78° (C = 0.68 in water)

Chromatography in solvent A showed 2,3-di-O-methyl galactose.

Subfraction 6(b) 3-O-methyl-L-rhamnose.

$[\alpha]_D$ = +35° (C = 0.21 in water)

Periodate oxidation and chromatography of the oxidation products gave a pattern of spots indicative of 3-O-methyl rhamnose.

The syrup crystallised when seeded with 3-O-methyl-L-rhamnose m.p. and mixed m.p. = 111-114°.

Fraction 7 (598 mg.)

R_G = 0.50, 0.45.

$[\alpha]_D$ = +81° (C = 2.1 in water)

Chromatography in solvent A showed 2,3-di-O-methyl-D-galactose and a relatively small amount of mono-O-methyl-arabinose.

Periodate oxidation and chromatography of the products indicated 2,3-di-O-methyl galactose.

Treatment of the syrup with aniline in dry ethanol gave a crystalline derivative, which was recrystallised twice from acetone, m.p. 139-140°. An X-ray powder photograph of the derivative was identical to that obtained from authentic 2,3-di-O-methyl-N-phenyl-D-galactosylamine (m.p. 154-5°).

The syrup (400 mg.) was fractionated on a charcoal : celite column by linear gradient elution with 0 - 2% butan-2-one into two chromatographically pure fractions. The fraction R_G = 0.47 was eluted from the column by a lower concentration of butan-2-one than the fraction R_G = 0.50.

Subfraction (a) (40. mg.)

R_G = 0.45

$[\alpha]_D$ = +98° (C = 0.5 in water)

Demethylation gave arabinose.

Periodate oxidation and chromatography of the oxidation products gave methoxymalondialdehyde only.

Attempts to prepare isopropylidene and toluene-p-sulphonylhydrazone derivatives failed.

The syrup (5.5 mg.) in water (5 ml.) was treated with 0.3 M-sodium periodate (3 ml.) and phosphate buffer (pH 8; 7 ml.). After 6 days formaldehyde in the solution was estimated by the chromotropic acid method

(78, 79). 0.79 mole of formaldehyde per mole of assumed mono-O-methyl pentose was released.

The sugar gave no reaction on a chromatogram with triphenyltetrazolium salt reagent.

Subfraction (b). (310 mg.)

The sugar was chromatographically identical to 2,3-di-O-methyl galactose.

Fraction 8. (146 mg.)

R_G = 0.50, 0.45, 0.41.

Chromatography in solvent A showed 2,3-di-O-methyl galactose, mono-O-methyl arabinose and rhamnose in the approximate ratio 3:1:1.

The component R_G = 0.41 was chromatographically identical to rhamnose in solvents A, B and C.

Fraction 9. (103 mg.) 2-O-methyl-D-galactose.

R_G = 0.33.

Chromatography in solvent A gave a single spot which was revealed by aniline oxalate but not by triphenyltetrazolium salt reagent.

Periodate oxidation and chromatography of the oxidation products gave methoxymalondialdehyde.

The sugar crystallised and was recrystallised from ethanol. m.p. = 148°, undepressed on admixture with 2-mono-O-methyl-D-galactose.

$[\alpha]_D$ = +54 ⟶ +82°.

Fraction 10 (52 mg.)

R_G = 0.33, 0.30.

The mixture was fractionated on Whatman 3MM filter paper in solvent H.

Sugar (a) (25 mg.)

R_G = 0.33.

Chromatography in solvent A showed 2-mono-O-methyl galactose.

The sugar crystallised and was recrystallised from ethanol. m.p. and mixed m.p. = 148°.

Sugar (b) 15 mg. 3-O-methyl-D-galactose.

$[\alpha]_D$ = +100°. (C = 0.11 in water)

Chromatography showed 3-mono-O-methyl galactose (R_G = 0.30).

Chromatography of the periodate oxidation products of the sugar showed mono-O-methyl pentose R_G = 0.40.

The syrup crystallised slowly when seeded with 3-mono-O-methyl-D-galactose. m.p. and mixed m.p. = 145-7°.

Fraction 11 (50 mg.)

Chromatography in solvents A and C showed 3-mono-O-methyl galactose and arabinose in approximately equal amounts.

Fraction 12 (180 mg.)

This fraction was eluted from the cellulose column with water.

Chromatography showed galactose only.

Oxidation of the sugar with nitric acid gave mucic acid m.p. = 215°.

INVESTIGATION OF THE METHANOLYSIS PRODUCTS OF METHYLATED METHYL PECTATE

Fully methylated methyl pectate (130 mg.) was treated with 4% methanolic hydrogen chloride in a sealed tube at 100° for 9 hr. The solution was neutralised with silver carbonate, filtered, treated with hydrogen sulphide, filtered and evaporated to dryness. The product was extracted with chloroform at room temperature to give a chloroform solution and chloroform-insoluble material.

Light petroleum (100-120°; 6 volumes) was added to the chloroform solution and a precipitate was isolated by centrifugation. The solution was evaporated to give a syrup (Fraction A) and the precipitate was dissolved in chloroform and concentrated to a syrup (Fraction B).

The chloroform-insoluble material was extracted with acetone at room temperature. The acetone solution was concentrated to a syrup (Fraction C) and acetone-insoluble material was dissolved in water and concentrated to a syrup (Fraction D).

Examination of fractions A, B, C and D.

Fraction A (67 mg.)

The syrup was hydrolysed with N-sulphuric acid for 2 hr. at 100°. Chromatography of the hydrolysate in solvents A and D showed the following methylated neutral sugars:-

2,3,5-tri-O-methyl arabinose

2,3,4,6-tetra-O-methyl galactose

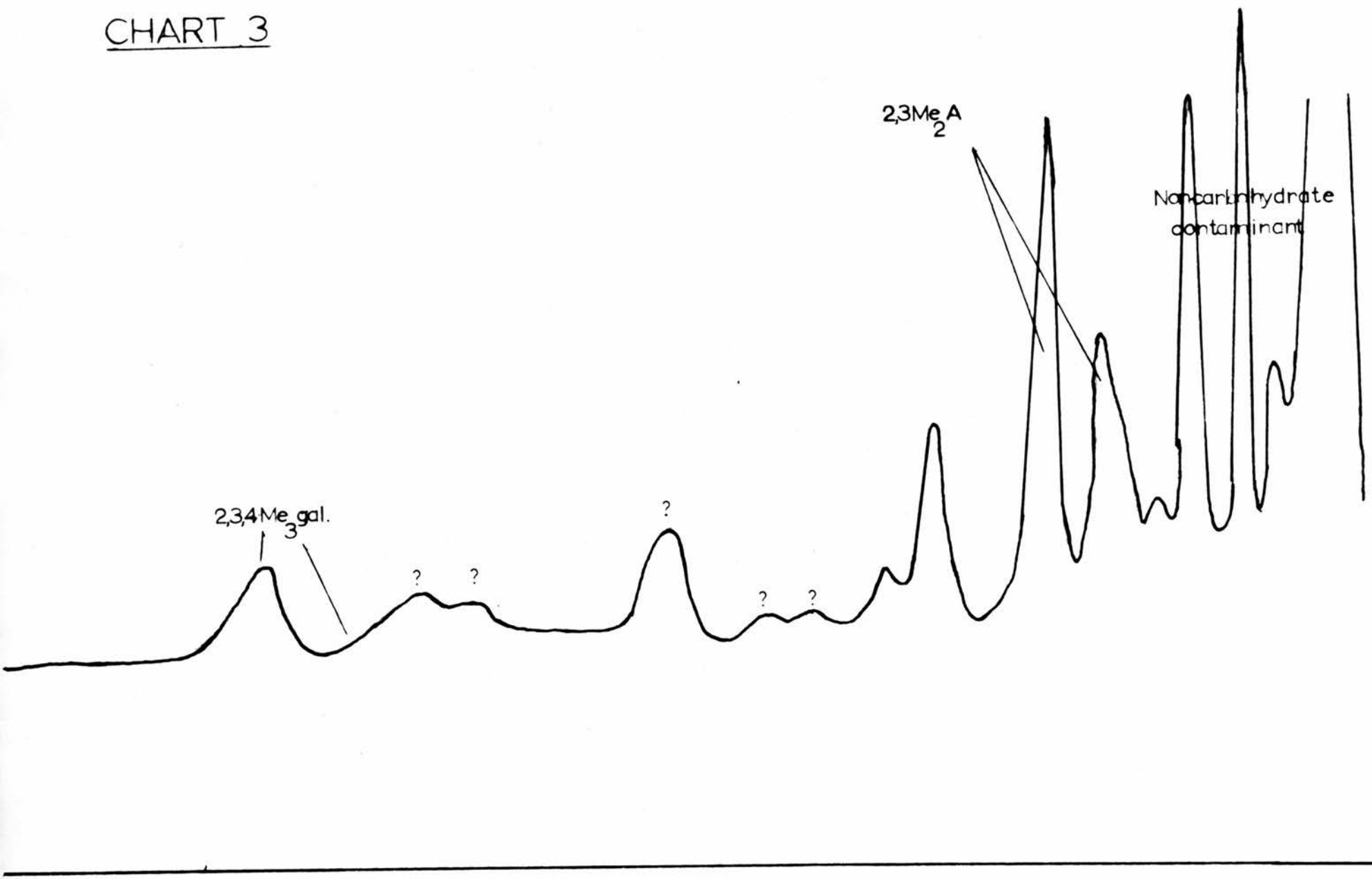
3,4-di-O-methyl rhamnose

2,3-di-O-methyl arabinose

2,3,4-tri-O-methyl galactose

mono-O-methyl arabinose

CHART 3



A mixture of the sugars 2,3-di-O-methyl arabinose and 2,3,4-tri-O-methyl galactose was separated from the syrup by chromatography on Whatman 3MM filter paper in solvent A, and was examined by means of vapour-phase chromatography by Dr. C.T. Bishop. Chart III shows the vapour-phase chromatogram of the methyl glycosides of the components of the mixture. It shows peaks corresponding to 2,3-di-O-methyl-L-arabinose and 2,3,4-tri-O-methyl-D-galactose and also a number of unknown materials in relatively small amount.

Fractions B. (28 mg.), C (30 mg.) and D (10 mg.)

Each fraction was hydrolysed with N-sulphuric acid at 100° for 4 hr. Chromatography of the hydrolysates in solvent A showed material of R_G = 0 - 0.1 and in solvent C showed material of R_F = 0.55 and R_F = 0.06 - 0.30.

The hydrolysates of fractions B, C and D were combined and fractionated on Whatman 3MM filter paper in solvent C.

Sugar of R_F = 0.55 (solvent C).

The syrup was treated with 4% methanolic hydrogen chloride at 100° for 3 hr., reduced with lithium aluminium hydride in dry tetrahydrofuran and hydrolysed with N-sulphuric acid for 2 hr. at 100°. Chromatography of the product in solvent A showed 2,3-di-O-methyl galactose.

Sugar mixture of R_F = 0.06 - 0.30 (solvent C).

The syrup was treated with 4% methanolic hydrogen chloride at 100° for 3 hr., reduced with lithium aluminium hydride and hydrolysed with N-sulphuric acid for 4 hr. at 100°. Chromatography of the product in solvent A showed 2,3-di-O-methyl galactose and also 3,4-di-O-methyl rhamnose and 2,3,4-tri-O-methyl galactose.

OTHER CARBOHYDRATE CONSTITUENTS OF LUCERNE

Mono- and Oligosaccharides

The syrup containing mono- and oligosaccharides extracted from lucerne by hot 80% ethanol was dissolved in water and placed on a charcoal:celite (1:1) column. The column was eluted with water, with water containing 2.5% ethanol (Fraction A) and with water containing 8% ethanol (Fraction B).

Fraction A

The 2.5% ethanol solution was concentrated, freeze dried onto cellulose and packed onto a cellulose column. The column was eluted with butan-1-ol, 50% saturated with water, to give sugars chromatographically identical to fructose, glucose and sucrose in solvent B.

D-fructose

The sugar (15 mg.) was added to 2 ml. of a mixture of dry acetone (10 ml.) and concentrated sulphuric acid (1 drop). The mixture was shaken for 5 hr. and then treated with water (4 ml.) and sufficient sodium carbonate to make the solution alkaline, and was then extracted with chloroform (3 x 10 ml.). The chloroform extracts were dried over anhydrous sodium sulphate and evaporated to dryness. The product was crystallised from petroleum ether (60-80°).

M.p. = 119° undepressed on admixture with 1:2,4:5-diisopropylidene-D-fructose.

D-glucose (80).

The sugar (50 mg.) was dissolved in water (1 drop) and treated with 2:4-dinitrophenylhydrazine (50 mg.) in ethanol (2 ml.) under reflux for 12 hr. The solution was evaporated to dryness and extracted with ethyl acetate. The residue was crystallised from 95% ethanol.

m.p. = 120-122° undepressed on admixture with D-glucose-2,4-dinitrophenylhydrazone.

Sucrose

The sugar (10 mg.) was hydrolysed; chromatography of the hydrolysate showed glucose and fructose.

The sugar (50 mg.) was dissolved in pyridine (1 ml.) and treated with acetic anhydride (2ml.) After 48 hr. the solution was treated with water and extracted with chloroform (3 x 5 ml.). The chloroform extracts were washed with a solution of sodium bicarbonate, dried over anhydrous sodium sulphate and evaporated to dryness. The product was crystallised from aqueous methanol.

m.p. and mixed m.p. with sucrose octaacetate = 70-72°.

Fraction B.

The 8% ethanol solution was evaporated to a syrup which contained fructose, glucose, sucrose and raffinose. The syrup was dissolved in water, freeze dried onto cellulose powder and packed onto a cellulose column. The column was eluted with ethyl acetate : butan-1-ol : pyridine : water (5 : 5 : 4 :3) to give a fraction containing raffinose only.

Hydrolysis and chromatography of the sugar showed galactose, glucose and fructose.

The sugar (50 mg.) was dissolved in pyridine (1 ml.) and added to acetic anhydride (2 ml.). After 48 hr. water was added to the solution to give a precipitate which was dissolved in chloroform. The chloroform solution was washed with a solution of sodium bicarbonate, dried over anhydrous sodium sulphate and evaporated to dryness. The product was crystallised from aqueous methanol.

m.p. and mixed m.p. with raffinose hendecaacetate = 98°.

Polysaccharide material extracted by cold water

The polysaccharide (p.31) had the following properties:

$[\alpha]_D$	= +70°
Uronic acid anhydride (carbazole method)	= 11.2%
Methoxyl	= 0.72%
Crude protein (% Nitrogen x 6.25)	= 8%
Ash	= 25%

The polysaccharide was hydrolysed with N-sulphuric acid for 4 hr. at 100°. Chromatography of the hydrolysate showed galactose, arabinose, xylose and rhamnose.

Polysaccharide material extracted by hot water

The polysaccharide (p.31) had the following properties:

$[\alpha]_D$	= +38°
Uronic acid anhydride (carbazole method)	= 22.3%
Methoxyl	= 1.15%
Crude protein (% Nitrogen x 6.25)	= 11%
Ash	= 4%

The polysaccharide was hydrolysed (N-sulphuric acid, 4 hr., 100°). Chromatography of the hydrolysate showed galactose, arabinose, xylose, glucose and rhamnose.

Araban-rich polysaccharide

The polysaccharide (p.32) extracted from lucerne by lime-water, after pectin-type polysaccharides had been removed from the plant tissues, had the following properties:

$[\alpha]_D$ = -23°

Uronic acid anhydride (carbazole method) = 8.8%

Crude protein (% N x 6.25) = 23%

Ash = 5%

Hydrolysis (N-sulphuric acid, 4 hr., 100°) and chromatography showed the following sugars as constituents of the polysaccharide in the approximate ratios indicated.

Galactose (2)	Xylose (1)
Arabinose (4)	Rhamnose (1)

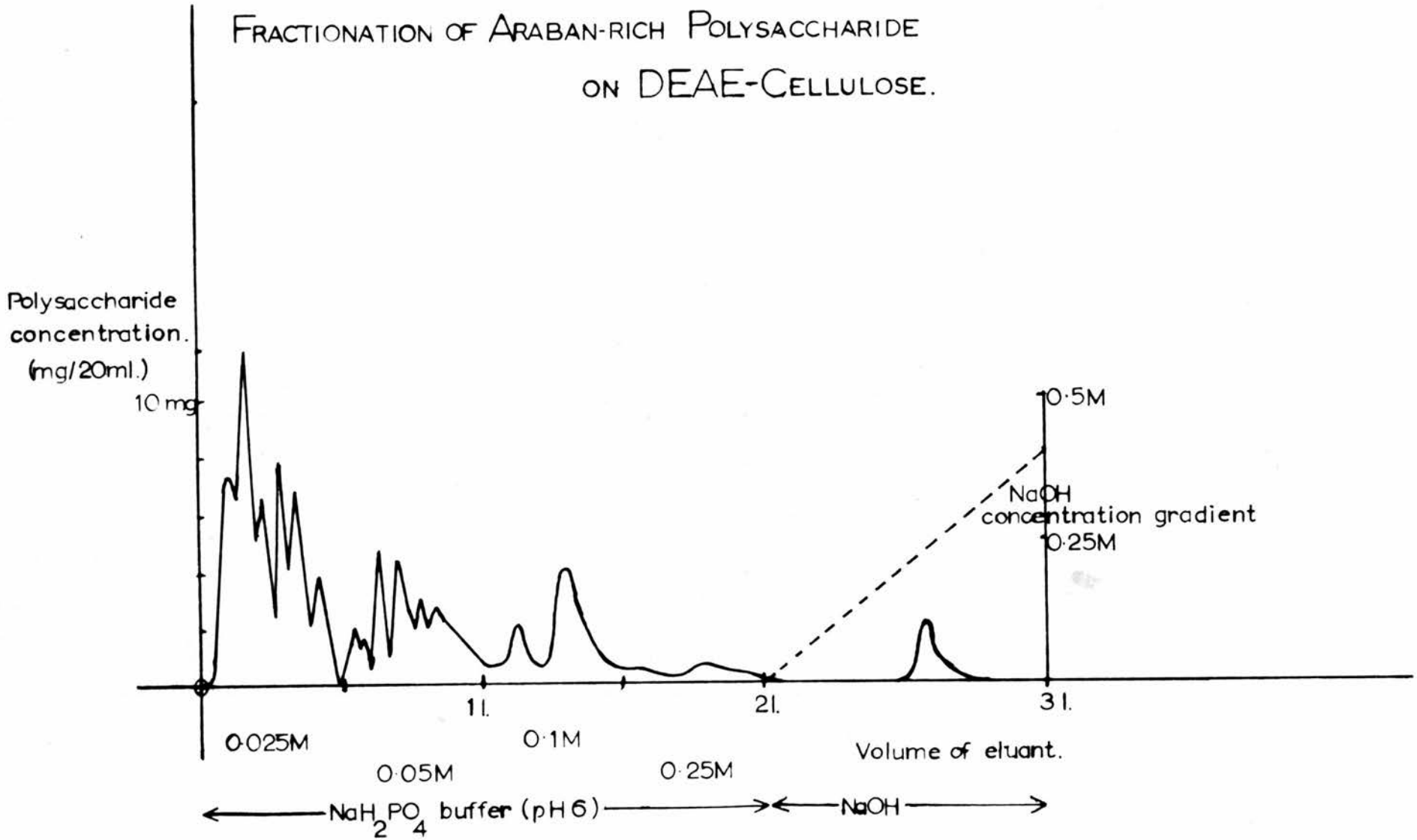
Fractionation of araban-rich polysaccharide

The polysaccharide (4 g.) was extracted with 70% ethanol under reflux for 24 hr. The 70% ethanol-insoluble fraction was dissolved in water and polysaccharide (1.5 g.) was precipitated by the addition of acetone (8 volumes), washed with acetone and ether, and dried in air and over phosphorous pentoxide under reduced pressure. The 70% ethanol solution was poured into acetone (4 volumes) and the precipitated polysaccharide was washed with acetone and ether and dried in air and over phosphorous pentoxide under reduced pressure.

The fractionated polysaccharides had the following properties:

	70% ethanol soluble fraction	70% ethanol insoluble fraction
$[\alpha]_D$	-50°	+9°
Uronic acid anhydride	4.7%	15.9%
Crude protein	37.3%	16.9%
Ash	3%	5%

Chromatography of the hydrolysis products of the two fractions


FRACTIONATION OF ARABAN-RICH POLYSACCHARIDE
ON DEAE-CELLULOSE.
Polysaccharide concentration. (mg/20ml.)
10 mg
0·5M
NaOH concentration gradient
0·25M
1l.
2l.
3l.
0·025M
0·05M
0·1M
0·25M
Volume of eluant.
NaH2PO4 buffer (pH 6)
NaOH

showed the following sugars in the ratios indicated.

70% ethanol-soluble fraction	70% ethanol-insoluble fraction
Galactose (2)	Galactose (2)
Arabinose (4)	Arabinose (1)
Xylose (1)	Xylose (4)
Rhamnose (1)	Rhamnose (1)

The 70% ethanol soluble polysaccharide (300 mg.) was dissolved in water and added to a DEAE cellulose column (3 x 30 cm) in the phosphate form. The column was eluted with the following concentrations of sodium dihydrogen phosphate buffer (pH 6) - (a) 0.025M; 500 ml. (b) 0.05M; 500 ml. (c) 0.1 M; 500 ml. (d) 0.25M; 500 ml.

Fractions (20 ml.) were collected automatically and the concentration of polysaccharide in each fraction was determined by the anthrone method (74). Then acidic polysaccharide was eluted from the column by a gradient of 0 - 0.4 N sodium hydroxide (1 l.) and the concentration in each fraction determined by the carbazole method (69).

The plot of polysaccharide concentration against volume and type of eluant is given.

DISCUSSION

Extraction

Carbohydrate constituents were extracted from lucerne by a succession of solvents, as shown below:-

Lucerne
↓ 80% ethanol
Residue A + mono- and oligosaccharides
↓ cold water
Residue B + pectin-type polysaccharide
↓ hot water
Residue C + pectin-type polysaccharide
↓ hot 0.5% ammonium oxalate solution
Residue D + Ammonium pectate
↓ hot saturated lime-water.
Residue + Araban-rich polysaccharide

The solvents chosen for the extractions were least liable to cause degradation of carbohydrates and so the constitution of the materials extracted and examined would be as near representative of materials present in the plant tissues as possible.

Carbohydrate constituents of Lucerne other than Pectic Acid.

The mixture of mono- and oligosaccharides extracted from lucerne was fractionated on charcoal and cellulose columns. Four components fructose, glucose, sucrose and raffinose were isolated and characterised. These sugars are common constituents of many plants.

Crude polysaccharides were isolated from residue A by extraction first with cold water and then with hot water. The polysaccharides resembled pectic substances in so far as they contained a relatively high proportion of uronic acid, much of which was esterified; they gave on hydrolysis, galactose arabinose, xylose and rhamnose and they had positive optical rotations.

No fructose-containing polysaccharide was obtained from lucerne, which is in contrast to the grasses many of which have been shown to contain fructosan. Moreover glucose-containing polysaccharides were not found to be abundant in lucerne.

After pectic substances had been extracted from lucerne, the residue was treated with hot lime water to yield araban-rich polysaccharide. This polysaccharide had a negative optical rotation, gave on hydrolysis arabinose,galactose, xylose and rhamnose, and contained uronic acid. The crude polysaccharide was fractionated by extraction with 70% ethanol. The material which was soluble in 70% ethanol contained a higher proportion of arabinose and much less xylose than the insoluble fraction, and the optical rotations were markedly different - the 70% ethanol soluble polysaccharide had a high negative rotation in contrast to the low positive optical rotation of the insoluble fraction. The 70% ethanol-soluble polysaccharide was added to a DEAE cellulose column. The column was eluted with increasing concentrations of phosphate buffer and then by gradient elution with 0 - 0.4 N sodium hydroxide. A plot of polysaccharide concentration in the eluant against volume of eluant showed that the polysaccharide

material is probably a complex mixture of polysaccharide which is eluted from DEAE cellulose by sodium hydroxide (0.2 - 0.3 N). The properties of the 70% ethanol soluble polysaccharide show that it is not unlike other araban-rich polysaccharides generally associated with pectin. It is unlikely that the isolated polysaccharide is wholly representative of material within the plant tissues because hot lime water is liable to cause substantial alkaline degradation of carbohydrate material. This possibly accounts for the fact that the polysaccharide is readily soluble in water and yet water extracts of lucerne failed to remove it from the plant tissues.

Lucerne Pectic Acid.

The ammonium pectate isolated from lucerne residue C by treatment of the residue with hot ammonium oxalate solution was virtually free from non-carbohydrate material. The optical rotation and the constituent sugars of the polysaccharide showed that it resembled other pectic substances of high galacturonic acid content.

Fractionation of Lucerne pectic acid.

Ammonium pectate was extracted with 70% ethanol under reflux. After several days no polysaccharide was detected in solution and the insoluble polysaccharide remained unchanged. Thus it seems likely that arabinose associated with lucerne pectic acid is not in the form of the highly branched neutral arabans which have been shown to be extracted from many pectic substances by 70% ethanol[21, 22].

Addition of calcium chloride to an aqueous solution of ammonium pectate gave complete precipitation of polysaccharide material from solution. The polysaccharide regenerated by treating

the insoluble calcium pectate with ammonium oxalate, was unchanged. Although the possibility of total coprecipitation cannot be overlooked it is to be expected that neutral polysaccharide would remain in solution under these conditions. Precipitation of pectin from solution as a calcium salt has been reported as a means of isolating a 1-4 linked galactan from white lupin seed pectin[30].

Similarly the action of hot lime-water on lucerne ammonium pectate caused precipitation of calcium pectate but failed to retain any neutral polysaccharide in solution.

The formation of a complex between a polysaccharide and cetavlon (cetyl trimethyl ammonium bromide) has been used in many cases to resolve mixtures of polysaccharides[73]. Cetavlon precipitates acidic polysaccharides from dilute solutions of their alkaline salts and treatment of the precipitates with acetic acid regenerates the polysaccharide. A complex of cetavlon and lucerne pectic acid formed readily and the polysaccharide which was regenerated by treating the complex with acetic acid showed virtually no change in optical rotation, uronic acid content and constituent sugars released by acid hydrolysis.

Lucerne pectic acid prepared by treating ammonium pectate with ion-exchange resins was placed on a column of DEAE cellulose in the phosphate form. Elution of the column with an increasing concentration of phosphate buffer gave very small amounts of neutral polysaccharide estimated by the anthrone method. Gradient elution of the column with sodium hydroxide solution gave a concentrated solution of polysaccharide which was eluted by 0.09 - 0.16 N sodium hydroxide. Examination of eluant fractions by the carbazole method

showed that this was the only major acidic polysaccharide fraction eluted, and a plot of polysaccharide concentration against volume of eluant showed a single large peak. The polysaccharide was isolated from the fraction by precipitation with acetone and was hydrolysed. The hydrolysate showed galactose arabinose, rhamnose, fucose, xylose, 2-O-methyl fucose and 2-O-methyl xylose. Little is known of the characteristics of DEAE cellulose for polysaccharide fractionation but it is the experience of other workers[40] that fractionation of neutral and acidic polysaccharides is readily achieved on the cellulose derivative.

The evidence provided by the failure to fractionate lucerne pectic acid by different fractionation techniques suggests that the association between the acidic and neutral sugars in the pectin is very close and points to the possibility that the neutral sugars are an integral part of acidic polysaccharide in pectin.

DEAE cellulose chromatography of the pectin suggests that the polysaccharide is virtually homogeneous.

Hydrolysis of Lucerne Pectic Acid.

Ammonium pectate was hydrolysed with sulphuric acid and the product was fractionated on a cellulose column. The major fractions of chromatographically pure sugars were isolated and the sugars were characterised as:-

D-galactose
L-arabinose
L-rhamnose

Both D-galactose and L-arabinose have been known for a long time to be associated with pectin and it has been shown recently that L-rhamnose is probably a component of pectic substances[39,25].

A relatively large amount of ammonium pectate was hydrolysed

under conditions favouring the production of aldobiouronic acids. Partially degraded pectin was precipitated by the addition of acetone to the hydrolysate. The acetone:water soluble sugar mixture which contained acidic and neutral sugars was fractionated on an anion-exchange resin column. The neutral sugar fraction was further fractionated on a charcoal/celite column. Elution of the charcoal column with water gave a mixture of monosaccharides and elution with water containing butan-2-one gave a mixture of sugars which was fractionated by paper chromatography. The following sugars were separated and identified

L-fucose
2-O-methyl-D-xylose
2-O-methyl-L-fucose

No evidence has been reported recently to suggest that L-fucose is a constituent of pectic substances but 2-O-methyl-D-xylose and 2-O-methyl-L-fucose have been reported present in polysaccharides of a pectic nature[39,25]. It has been suggested that in the alkali-stable pectic material of sugar beet, 2-O-methyl-D-xylose is linked to galacturonic acid to form an aldobiouronic acid unit[25]. Vigorous hydrolysis of lucerne pectic acid gave an increase in the concentration of 2-O-methyl-L-fucose as judged by chromatography. Thus it would seem that the linkage of this sugar within the polysaccharide is acid resistant and if this is so it is likely that the sugar is in the form of an aldobiouronic acid unit.

Acidic sugars were eluted from the anion exchange resin column by a water-formic acid gradient to give mixtures of aldobiouronic acids, chromatographically pure galacturonic acid and di- and trigalacturonic acids. Aldobiouronic acid mixtures were fractionated by chromatography

on filter paper.

A relatively large amount of an aldobiouronic acid was isolated and characterised by methylation study. Hydrolysis, and methanolysis, reduction and hydrolysis showed that the sugar was composed of galacturonic acid and rhamnose. The sugar was methylated by the Haworth method and the fully methylated acid was obtained as a crystalline product which was reduced with diborane and hydrolysed. The hydrolysis product contained two methylated sugars which were separated by chromatography on filter paper and characterised as:-

2,3,4-tri-O-methyl-D-galactose

3,4-di-O-methyl-L-rhamnose

On the basis of methylation study and the high positive rotation of the methylated acid the oligosaccharide was assumed to be 2-O- α -D-galacturonosyl-L-rhamnose. Although this sugar has not been reported as a constituent of pectic substances it is a common structural unit of many gums and mucilages containing galacturonic acid.

Two other aldobiouronic acids in relatively small amount were isolated but complete characterisation of the sugars was not achieved. Evidence based on hydrolysis, and methanolysis, reduction and hydrolysis suggested that one aldobiouronic acid was composed of galacturonic acid and fucose and the other of galacturonic acid and galactose as judged by chromatography. Small amounts of other sugars associated with hydrolysates of these two aldobiouronic acids could have arisen from polysaccharide contaminant extracted from the chromatogram paper[90] from which the aldobiouronic acids were extracted.

An aldotriouronic acid containing galacturonic acid and rhamnose was isolated and shown to be chromatographically free of other sugars. Reduction of the sugar with potassium borohydride and subsequent

subsequent hydrolysis gave galacturonic acid, rhamnose and rhamnitol thus indicating that a rhamnose unit was functioning as reducing end group. Hydrolysis of the bromine oxidation product of the sugar was shown by chromatography to contain rhamnose which could have arisen from incomplete oxidation of rhamnose reducing end group or from a rhamnose unit in some other position in the molecule. Mild hydrolysis of the sugar gave a product which contained rhamnose, galacturonic acid and a sugar chromatographically identical to 2-O-galacturonosyl-rhamnose. A molecular weight determination by the hypoiodite oxidation method suggested that the sugar was a trisaccharide. The value was high but complete oxidation of reducing sugars with the mannose configuration has been shown difficult to achieve[76]. The galacturonic acid content of the sugar determined by the carbazole method corresponded to one galacturonic acid unit in each molecule. The sugar had a high positive rotation. The above evidence suggests that the sugar is a trisaccharide composed of galacturonic acid and two rhamnose units, one of which represents the reducing end group, also that the linkage between galacturonic acid and one of the rhamnose units is 1 ⟶ 2. On the basis of methylation study of lucerne pectic acid it can be said that a linkage of galacturonic acid to rhamnose is confined to either position C_2 or C_4 of the rhamnose unit. Three possible structures of the aldotriouronic acid can be given as shown below.

(a) Rh 1 ——— Gal A1 ——— 2 Rh.

(b) Gal A1 ——— 2Rh 1 ——— (2) or (4) Rh

(c) Gal A1 ——— 2Rh
4
|
Rh 1

Enzyme Degradation of Acid-degraded Pectin.

The acetone:water (1:1) insoluble material from the hydrolysis of ammonium pectate with N-sulphuric acid (p.38) was dissolved in water and adjusted to pH 4 with glacial acetic acid. Light's hemicellulase was added to the solution at 20°. After 8 hr. acetone (1 volume) was added to the solution which was then filtered and evaporated to dryness.

Chromatography of the product in solvents C and G showed galacturonic acid and di- and trigalacturonic acids. No neutral monosaccharides were apparent. The galactobiose was separated from galactose by chromatography on filter paper, and was shown to be chromatographically and ionophoretically identical to 4-O-α-D-galactopyranosyl-D-galactose prepared by Jones and Reid (37) from digalacturonic acid isolated as an enzyme degradation product of pectin.

Enzyme degradation of the partially degraded material from the acid hydrolysate of ammonium pectate gave galacturonic acid, di- and trigalacturonic acid and no neutral monosaccharides which suggests that in lucerne pectic acid, as in other pectic substances there are chains of galacturonic acid units relatively resistant to acid hydrolysis.

Enzyme Degradation of Lucerne Pectic Acid.

Ammonium pectate was treated with anion and cation exchange resins and the pectic acid produced was degraded with a commercial pectinase preparation. The product contained partially degraded

material, neutral sugars, galacturonic acid and di- and trigalacturonic acid.

Chromatography of the mixture, to detect neutral sugars, showed galactose, arabinose and rhamnose and none of the other neutral sugars produced by acid hydrolysis of the pectic acid. However the partially degraded material was shown by hydrolysis and chromatography to contain a wide variety of sugars, namely: galacturonic acid, galacturonosyl-rhamnose, galactose, arabinose, xylose, fucose, rhamnose, mannose, 2-O-methyl xylose and 2-O-methyl fucose.

Before attempting to separate di- and trigalacturonic acid from the mixture of acidic and neutral sugars it was necessary to remove the large amount of galacturonic acid which would interfere with such a separation. A solution of the mixture was neutralised with calcium carbonate and a precipitate of the calcium salts of di- and trigalacturonic acid together with some calcium galacturonate was obtained by the addition of ethanol to the solution. The alcoholic solution was evaporated to dryness and the product was extracted with methanol to give a methanolic solution which contained arabinose, galactose, rhamnose and a little galacturonic acid. The methanol insoluble residue composed mainly of calcium galacturonate was treated with cation-exchange resin and the product was fractionated by chromatography on anion-exchange resin. A pure fraction of galacturonic acid was isolated and characterised.

Di- and trigalacturonic acids were separated by paper chromatography but elution of the sugars from the chromatograms with hot water gave complex mixtures which included galacturonic and digalacturonic

acids. It seems likely that hot water can cause considerable degradation of di- and trigalacturonic acid.

The mixture of sugars produced by elution of the digalacturonic acid was treated with benzyl alcohol saturated with dry hydrogen chloride, reduced with lithium aluminium hydride in tetrahydrofuran and hydrogenated. The product was fractionated by chromatography and a sugar chromatographically and ionophoretically identical to 4-O- ∝ -D-galactopyranosyl-D-galactose was isolated.

Methylation study of lucerne pectic acid.

Lucerne ammonium pectate was partially methylated by treatment with Haworth's reagents and the partially methylated sodium pectate was isolated, converted to the silver salt and methylated fully by treatment with Purdie's reagents. The methylated methyl pectate was heated at 100° with methanolic hydrogen chloride, the product was reduced with lithium aluminium hydride in tetrahydrofuran and the reduction product was hydrolysed. The following methylated sugars were isolated from the hydrolysate and characterised.

2,3,4,6-tetra-O-methyl-D-galactose

2,3,4-tri-O-methyl-D-galactose

2,3-di-O-methyl-D-galactose

2-O-methyl-D-galactose

3-O-methyl-D-galactose

2,3,5-triO-methyl-L-arabinose

2,3-di-O-methyl-L-arabinose

2,(?)-O-methyl-L-arabinose

3,4-di-O-methyl-L-rhamnose

3-O-methyl-L-rhamnose

2,3,6-Tri-O-methyl-D-galactose was not found in the mixture and vapour phase chromatography indicated that the sugar was not present.

A relatively small amount of methylated methyl pectate was treated with methanolic hydrogen chloride at 100° and the product was fractionated by extraction and precipitation with various solvents. A fraction containing mainly methylated neutral sugars was hydrolysed and chromatography of the hydrolysate showed

2,3,4,6-tetra-O-methyl-D-galactose

2,3,4-tri-O-methyl-D-galactose

2,3,5-tri-O-methyl-L-arabinose

2,3-di-O-methyl-L-arabinose

2,(?)-O-methyl-L-arabinose

3,4-di-O-methyl-L-rhamnose

Vapour phase chromatography confirmed the presence of 2,3,4-tri-O-methyl-D-galactose and 2,3-diO-methyl-L-arabinose.

Other fractions were hydrolysed in aqueous solution and separated by chromatography into two fractions. Methanolysis, reduction and hydrolysis of one fraction gave 2,3-di-O-methyl galactose and some 2,3,4-tri-O-methyl galactose and 3,4-di-O-methyl rhamnose as judged by chromatography. Methanolysis, reduction and hydrolysis of the other fraction gave 2,3-di-O-methyl galactose.

Methylated galactose derivatives isolated from the reduced methylated methyl pectate could have arisen either from galactose

units or from galacturonic acid units when no substituent was present at position C_6, and from galactose only when a substituent did occur at position C_6.

2,3,4,6-Tetra-O-methyl-D-galactose must have been derived from non-reducing galactose end group. 2,3,4-Tri-O-methyl-D-galactose was obtained from both reduced and non-reduced methylated methyl pectate and so it is evident that although non-reducing galacturonic acid end group could give rise to this sugar much of it must have been produced by methylation of 1-6 linked galactose units. No 2,3,6-tri-O-methyl-D-galactose was detected and so indicated that no 1-4 linked galactose units were present in the pectic acid and thus shows that 1-4 linked galactan of the type found in lupin seed pectin[30] is not a component of lucerne pectic acid. A major difference between the reduced and non-reduced methylated pectic acid was that the former contained 2,3-di-O-methyl-D-galactose units whereas the latter contained none, thus indicating that the sugar was derived from galacturonic acid units and consequently a large proportion of the pectic acid must be composed of 1-4 linked galacturonic acid units. 2- and 3-O-methyl-D-galactose indicate that the 1-4 linked galacturonic acid chains have a number of galacturonic acid branch points. It is not possible to determine to what extent these chains are branched since some mono-O-methyl galactose could arise as a product of incomplete methylation.

No di-O-methyl-D-galactose units were detected in methylated lucerne pectic acid and the relative amounts of 2,3,4-tri-O-methyl-

D-galactose were such that no straight chain galactan can be formulated. It is most likely that they represent short chains of galactose units linked at branch points to a polygalacturonic acid chain.

The isolation of 3,4-di-O-methyl-L-rhamnose from the methylated pectin is consistent with the production of a relatively large amount of 2-O-D-galacturonosyl-L-rhamnose by acid hydrolysis of the pectic acid. That rhamnose also acts as a branch point in the pectin is shown by the presence of 3-O-methyl-L-rhamnose units in the methylated material.

The structural significance of arabinose in pectic acid is not clearly defined by the methylation study but since it is unlikely that a neutral araban is present in lucerne pectic acid it can be said that if side chains of arabinose units are attached to polygalacturonic acid they will be of a branched nature.

That the many different sugar units present in lucerne pectic acid are intimately associated is evident from the results of attempted fractionation of the polysaccharide, in fact these results suggest that neutral sugars are linked to acid polysaccharide chains. Hydrolysis of the pectin gave evidence that rhamnose is definitely involved in the structure of an acidic polysaccharide, and possibly galactose and fucose also. The manner in which 2-O-galacturonosyl rhamnose is involved in the structure of the pectic acid cannot be proposed precisely. It is

It is possible that such a unit could be dispersed within a galacturonic acid chain as shown below.

- 4 Gal A 1 - 4 Gal A 1 - 2 Rh 1 - - - - - 4 Gal A 1 -

It has been reported[54] that rhamnose units are dispersed within a polysaccharide chain of 1-4 linked galacturonic acid and galactose units in Khaya grandifolia gum.

Methylation study indicated that galacturonic acid units in the pectic acid are 1-4 linked, probably in the form of long chains, and also indicated the mode of linkage of other sugars. Evidence showing the order in which sugar units occur in the polysaccharide is not given by methylation study. It was hoped that enzyme degradation of the pectin would provide this evidence. Ideally an enzyme system capable of hydrolysing only the 1-4 links of polygalacturonic acid would yield fragments of galacturonic acid, di- and trigalacturonic acids to which any side chains of acidic or neutral sugars would be attached. Examination of these fragments would give detailed knowledge of the structural significance of neutral sugars linked to polygalacturonic acid. The crude pectinase used to degrade lucerne pectic acid was not a specific enzyme system and caused considerable hydrolysis of neutral sugar units. No acidic oligosaccharides containing neutral sugar units were produced by enzymic degradation of the pectin.

SECTION II.

REDUCTION OF CARBOHYDRATE CARBOXYL GROUPS BY DIBORANE

Introduction

Diborane (B_2H_6) is a gas (b.p. -92.5°), stable in dry air and is not spontaneously inflammable. It is rapidly hydrolysed by water to boric acid and hydrogen.

The ability of diborane to reduce aldehydes and ketones has been known for a long time[81]. Recently the reducing action of diborane on other types of functional group has been investigated by Brown[82]. It has been shown to exhibit markedly different selectivity than the complex metal hydrides. Thus carboxylic groups are reduced rapidly, whereas ester groups are reduced only slowly, by diborane.

There is a wide variety of methods for the synthesis of diborane[56].

Lithium and sodium borohydrides react readily with hydrogen chloride in a suitable solvent to liberate diborane. Many other metallic

$$2\ LiBH_4 + 2\ HCl \longrightarrow 2\ LiCl + 2\ H_2 + B_2H_6$$

borohydrides react in a similar manner.

The reaction of sodium or lithium hydride with boron trifluoride etherate serves for the preparation of diborane.

$$6\ NaH + 8\ BF_3 \xrightarrow{\text{Ether}} 6\ NaBF_4 + B_2H_6$$

Also diborane is generated by the action of lithium aluminium hydride, or lithium borohydride, on lithium borofluoride.

$$LiBF_4 + 3\ LiBH_4 \longrightarrow 2\ B_2H_6 + 4\ LiF.$$

Probably the most convenient method for the generation of diborane is to react either boron trifluoride or boron trichloride with sodium or

lithium borohydride in an ether-type solvent.

$$3\ LiBH_4 + BX_3 \xrightarrow{\text{Ether}} 2\ B_2H_6 + 3\ LiX$$

It has been suggested by Zweifel[83] that diborane could be used for the reduction of carbohydrate carboxylic acids.

EXPERIMENTAL

Generation of diborane

The apparatus used to generate diborane is illustrated. All parts are standard Quickfit units, modified where necessary to suit the requirements of the preparation.

Nitrogen, dried by passing through a column of granular magnesium perchlorate (Anhydrone) was introduced through the inlet-tube A to displace air and water vapour from the apparatus. Lithium borohydride (0.5 g.) in diglyme (10 ml.) was added to the flask and stirred by a magnetic stirrer. Tetrahydrofuran (40 ml.) was added to the receiver and acetone was added to the trap. Nitrogen was passed through the apparatus at a rate sufficient to give a fine stream of bubbles through the receiver solution. Boron trifluoride etherate (4.0 g.) in diglyme (10 ml.) was added to the dropping funnel, the top of which was connected to the nitrogen supply.

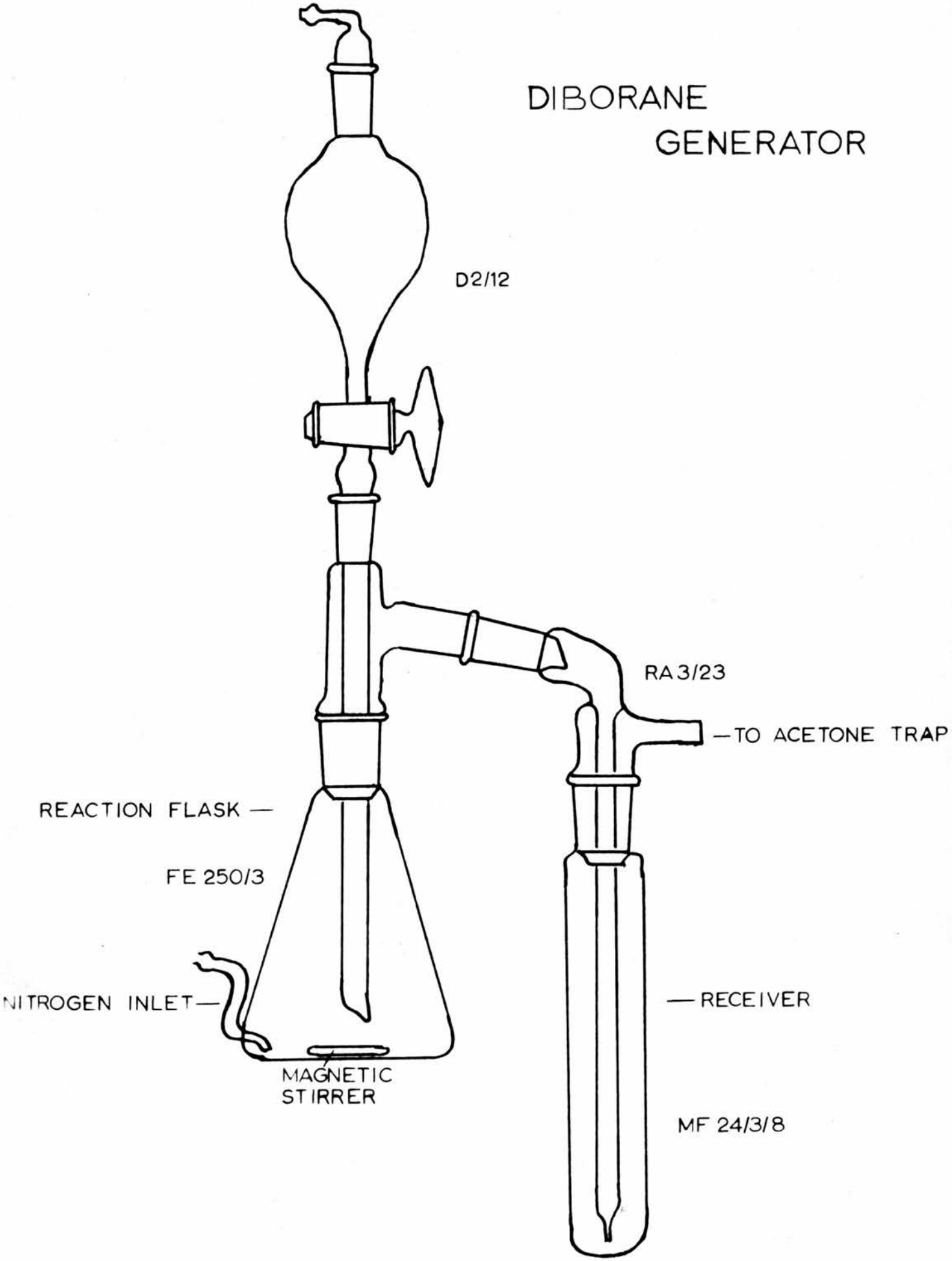
The boron trifluoride solution was added dropwise to the contents of the flask over a period of 1 hr, and the diborane generated was carried over in nitrogen to the receiver.

The solution of diborane in tetrahydrofuran was added to a solution of the carbohydrate material in tetrahydrofuran. Alternatively diborane was bubbled into a solution of the carbohydrate material in tetrahydrofuran.

Reduction of methyl-2,3,4-tri-O-methyl-β-D-glucuronoside

Methyl-2,3,4-tri-O-methyl-β-D-glucuronoside was prepared from

DIBORANE GENERATOR



glucuronic acid by the action of Haworth's reagents[84] and recrystallised from light petroleum (40-60°): ether. m.p. = 128°.

The sugar (0.65 g.) was dissolved in dry tetrahydrofuran (20 ml.) and treated with an excess of diborane in tetrahydrofuran (20 ml.). After 3 hr. excess diborane was destroyed by the addition of ethanol and water. The solution was evaporated to dryness and the product was dissolved in methanol. The methanol solution was evaporated to dryness thus removing boric acid. The treatment with methanol was repeated until all boric acid had been removed. The product was recrystallised from light petroleum (60-80°).

Yield of crystalline product = 0.52 gm.

m.p. 92° mixed m.p. 90° with authentic methyl-2,3,4-tri-O-methyl-β-D-glucoside (m.p. 89°).

The product was hydrolysed. Chromatography of the hydrolysate showed 2,3,4-tri-O-methyl glucose only.

Reduction of a methyl galactosyl uronic acid rhamnoside pentamethyl ether.

The methylated sugar (0.19 g.), prepared from galacturonosyl-rhamnose by treatment with Haworth's reagents, was dissolved in dry tetrahydrofuran (20 ml.) and treated with an excess of diborane in tetrahydrofuran. After 12 hr. ethanol and water were added to the solution and the mixture was evaporated to dryness. The product was treated with methanol to remove boric acid and was then hydrolysed (p. 42). Chromatography of the hydrolysate showed 2,3,4-tri-O-methyl galactose and 3,4-di-O-methyl rhamnose only. These sugars were separated and characterised (p. 42).

Yield of methyl galactosyl rhamnoside pentamethyl ether = 0.18 g.

Reduction of methylated Norway spruce xylan

The methylated xylan (0.20 g.) was treated with an excess of diborane in tetrahydrofuran. After 20 hr. diborane was destroyed by the addition of ethanol and water and the solution was evaporated to dryness. Boric acid was removed by treatment with methanol. Yield of product (0.18 g.).

The product and the non-reduced methylated xylan were treated separately with 2% methanolic hydrogen chloride in sealed tubes at 100° for 3 hr. The methanolysis products were hydrolysed with N-hydrochloric acid at 100° for 3 hr. Chromatography of the hydrolysates in solvents A, C and D showed that the non-reduced material contained a methylated aldobiouronic acid which was not apparent in the hydrolysate of the reduced material, that 2,3,4-tri-O-methyl glucose was a constituent of the reduced material but not of the non-reduced material, and also that the reduced material gave more mono-O-methyl-xylose than the non-reduced material when hydrolysed.

An uronic acid anhydride estimation by the Kaye and Kent method[85] on the reduced and non-reduced methylated xylans using glucurone as reference compound and methyl trimethyl glucoside to determine the correction factor gave:-

Non-reduced material	:	9% uronic acid anhydride
Reduced material	:	ca.0.4% uronic acid anhydride

Reduction of methyl-triacetyl-α-D-mannuronoside.

Methyl-D-mannuronoside which was contaminated with methyl D-mannoside was dissolved in pyridine:acetic anhydride (1:2). After 24 hours at room temperature the solution was diluted with water and after

3 hr. the solution was made acidic with N-sulphuric acid and extracted with chloroform. The extracts were evaporated to dryness and the product was treated with an excess of diborane in tetrahydrofuran. After 20 hr. the reduction product was isolated in the usual way and treated with methanol to remove boric acid. Hydrolysis of the product and chromatography of the hydrolysate in solvents B and C showed mannose only.

The reduction product crystallised and was recrystallised from ethanol.

m.p. = 185° and mixed m.p. = 183°

with authentic methyl- α -D-mannoside (m.p. 183°)

Attempted reduction of partially methylated pectic acid.

The partially methylated sisal pectic acid (OMe = 14%) was dissolved in tetrahydrofuran and treated with an excess of diborane in tetrahydrofuran. After ½ hr. a gel-like precipitate began to form in the solution and after 1 hr. the solution had gelled completely. After 20 hr. the product was isolated and boric acid was removed in the normal way. The product was treated with 2% methanolic hydrogen chloride for 3 hr. in a sealed tube at 100° and then with N-hydrochloric acid for 6 hr. at 100°. Chromatography of the hydrolysate showed methylated acidic material and some 2,3 di-O-methyl galactose not present in the hydrolysate of the non-reduced methylated pectic acid.

Attempted Reduction of acetylated gum ghatti.

Gum ghatti (5 g.) was dissolved in formamide (60 ml.) at 45-50°. Pyridine (60 g.) was added dropwise to the solution at 40-50° over a

period of 30 min. The solution was cooled to 30° and acetic anhydride (40 g.) was added dropwise over a period of 30 min. The solution was stirred at 30° for 5 hr., kept at room temperature for 20 hr, and was then poured into iced water (1 l.) containing 2% hydrochloric acid. The precipitated acetylated gum ghatti was washed with water and freeze-dried. The product was extracted with tetrahydrofuran. Light petroleum (60-80°) was added to the extracts to give a precipitate of acetylated material (0.89 g.) which was washed with light petroleum and ether.

The product was treated with diborane in tetrahydrofuran. A gel began to form in the solution almost immediately. After 20 hr. the product was isolated and treated with methanol. Hydrolysis and chromatography of the product showed a small amount of glucose in addition to other sugars present in the non-reduced polysaccharide.

The action of diborane with acetylated fructosan.

Diborane in tetrahydrofuran was added to a solution of acetylated fructosan in tetrahydrofuran. After 1 hr. the mixture had formed a gel similar to the gels produced by acetylated gum ghatti and partially methylated pectic acid in the presence of diborane in tetrahydrofuran.

Discussion

It has been shown that diborane is generated readily by the interaction of boron trifluoride etherate and lithium borohydride, and that the diborane gas generated can be collected in tetrahydrofuran.

Solutions of diborane were used to reduce completely methyl-2,3,4-tri-O-methyl-β-D-glucuronoside and methyl galactosyl uronic acid rhamnoside pentamethyl ether to the corresponding reduction products.

Methylated Norway spruce xylan was reduced with diborane. The methylated xylan[86] is composed of 1-4 linked-β-D-xylopyranose residues, every fifth residue carrying a terminal 4-O-methyl-glucuronic acid residue linked through position 2. Hydrolysis of the methylated xylan gives methyl ether derivatives of xylose and a methylated aldobiouronic acid containing mono-O-methyl xylose and tri-O-methyl glucuronic acid. After reduction, hydrolysis of the methylated xylan gave no methylated aldobiouronic acid, but produced, in addition to methyl ether derivates of xylose, tri-O-methyl glucose thus indicating that complete reduction had occurred. An estimation of uronic acid anhydride present in the reduced methylated xylan gave a percentage figure which falls within the range of experimental error of the method[85] used.

Acetylated methyl-D-mannuronoside when treated with diborane gave a product which was shown by hydrolysis and chromatography to be a derivative of mannose. The product was crystallised and had m.p. and mixed m.p. corresponding to methyl-α-D-mannoside. It was thus evident that reduction had taken place, and also that deacetylation had occurred under the conditions of the reduction.

Complete reduction of partially methylated pectic acid and acetylated gum ghatti was not achieved though it was evident that some

reduction had occurred. Partially methylated pectic acid after treatment with diborane gave by hydrolysis 2,3-di-O-methyl-galactose which was not produced by hydrolysis of the methylated pectic acid itself. Similarly reduced acetylated gum ghatti gave glucose by hydrolysis which must have been derived from glucuronic acid. Gum ghatti[87] contains glucuronic acid residues but no glucose residues.

A characteristic reaction of the partially methylated pectic acid and acetylated gum ghatti in the presence of diborane in tetrahydrofuran was that after a short time an insoluble gel-like precipitate of the polysaccharide derivative was formed. It is possible that diborane is unable to react with the material in this condition. The factors involved in the production of the precipate are not known. However it is known that deacetylation during the reaction may occur (cf. acetylated methyl mannuronoside) thus exposing free hydroxyl groups capable of complex formation with boric acid or other boron derivatives. Free hydroxyl groups would be available for such complex formation in partially methylated pectic acid.

Acetylated fructosan has been shown to give an insoluble precipitate in tetrahydrofuran in the presence of diborane thus showing that acidic groups may take no part in the precipitation reaction.

Diborane reduction of carboxyl groups in carbohydrates has been investigated simultaneously by Smith and Stephen[88]. They generated diborane *in situ* by adding borontrifluoride etherate to a solution of the carbohydrate material and sodium borohydride in diglyme. In this way they reduced methyl-α-D-galacturonoside, methyl 2,3,4-tri-O-methyl-α-D-galacturonoside and galactaric acid. They showed by chromatography

and determination of equivalent weights that mesquitic acid acetate and pectic acid acetate could be reduced almost completely, and that alginic acid propionate could be reduced extensively by diborane.

ACKNOWLEDGEMENTS

I wish to express gratitude to Professor E.L. Hirst for his interest and guidance and to Dr. G.O. Aspinall for the encouragement and invaluable advice he gave so willingly during the course of my work.

Thanks are due to Dr. C.E. Beevers for facilities for X-ray powder photography, Dr. C.T. Bishop, National Research Council, Ottawa for the vapour phase chromatograms and their interpretation, to Professor J.K.N. Jones for samples, and to the Agricultural Research Council and Imperial Chemical Industries Ltd. for maintainance grants.

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